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# Interaction between

# Glycogen Synthase Kinase-3 and Estrogen Receptor-α

# in ligand-dependent activation of the receptor

Presented by

**Jean GRISOUARD**

(Born in Dijon, France)

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Referees **Prof. Dr. Doris Mayer**

**Prof. Dr. Lutz Gissmann**

***To Mathieu Jourd'heuil,***

***To Felix Bub,***

*In Their Memory.....*

***To my Great Grand-Ma'***

*Bravery, Strength and Will.....*

***To my Family,***

*My Refuge.....*

***To Sandra,***

*My Love.....*

***To my Friends,***

*From my little village, to the next town and all over the world.....*

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# Zusammenfassung

Glycogensynthasekinase-3 (GSK-3), eine Serin/Threonin-Kinase mit Docking-Eigenschaften, reguliert zahlreiche zelluläre Prozesse. Zwei Isoformen, GSK-3α und GSK-3β, wurden beschrieben. GSK-3β stellt die Hauptform *in vivo* dar, sie spielt eine Schlüsselrolle bei der Regulation von Transkriptionsfaktoren inklusive der Steroidrezeptoren. Ziel der vorliegenden Arbeit war, die Rolle der GSK-3 bezüglich der Funktion von Estrogenrezeptor-α (ERα) in Brustkrebszellen zu entschlüsseln. Als experimentelles Modell wurden hauptsächlich MCF-7 Zellen, eine ERα-positive menschliche Brustkrebszelllinie, verwendet.

Silencing von GSK-3α und/oder GSK-3β durch Transfektion spezifischer siRNA-Sequenzen führte zur Degradation von ERα durch das Proteasom. Durch Verwendung des Proteasomeninhibitors MG132 konnten die ERα - Proteinspiegel in GSK-3 siRNA-transfizierten Zellen wiederhergestellt werden. Dies zeigt, dass GSK-3 den ERα stabilisiert und vor proteasomaler Degradation schützt. In einem weiteren experimentellen Ansatz wurde endogene GSK-3β durch Transfektion der Zellen mit microRNA - Konstrukten spezifisch ausgeschaltet; auch hier wurde das Silencing der GSK-3β von einer Reduktion des ERα-Proteinspiegels begleitet. In diesen Zellen konnten die ERα - Proteinspiegel durch Überexpression von Wildtyp- oder Kinase - inaktiver Xenopus-GSK-3β wiederhergestellt werden. Daraus ist zu schließen, dass die Docking-Eigenschaften der GSK-3 und nicht deren Kinaseaktivität für die ERα - Stabilisierung wichtig sind.

Behandlung von Zellen mit 17β-Estradiol führte zu rascher Phosphorylierung und Inaktivierung zytoplasmatischer GSK-3. Diese Phosphorylierung an GSK-3 führte zur Freisetzung von ERα aus dem GSK-3/ERα - Komplex und seine Translokation in den Zellkern. Dort wurde ERα durch aktive kernständige GSK-3β an Ser-118 phosphoryliert; diese Phosphorylierung ist essentiell für die volle Aktivierung von ERα. Behandlung der Zellen mit dem GSK-3 Inhibitor LiCl führte zur Hemmung der E2-induzierten Phosphorylierung an Ser-118, denselben Effekt zeigte die Reduktion des GSK-3 Spiegels im Zellkern nach GSK-3 Silencing. Diese Ergebnisse belegen, dass ein nukleärer Pool aktiver GSK-3, welcher durch Behandlung der Zellen mit E2 nicht phosphoryliert und inaktiviert wird, für die E2-induzierte Ser-118 Phosphorylierung von ERα nötig ist. Als Konsequenz der verminderten Ser-118 Phosphorylierung in GSK-3 siRNA - transfizierten Zellen wurde eine

signifikante Reduktion der transkriptionalen Aktivität des ERα beobachtet. Diese Aktivitätsminderung wurde sowohl durch ERE-abhängige Luziferase Reporter-Assays als auch durch Messen der Transkription der ERα - abhängigen endogenen Gene pS2 und Progesteronrezeptor durch quantitative Real-Time PCR nach E2-Behandlung in GSK-3 siRNA - transfizierten Zellen nachgewiesen. Weder die Ser-118 Phosphorylierung an ERα noch die ERα-Aktivität konnte durch Inkubation von GSK-3 siRNA - transfizierten Zellen mit MG132 wiederhergestellt werden, was die Bedeutung der nukleären GSK-3 für diese Prozesse unterstreicht. Weiterhin konnte durch Überexpression humaner GSK-3β in stabil mit einem ERE-kontrollierten Luziferase Reportergen transfizierten MCF-7 Zellen die Funktion der GSK-3 bei der E2-induzierten Aktivierung von ERα bestätigt werden. Durch Expression von GSK-3β - Mutanten, welche gegenüber geprimten GSK-3 - Substraten inaktiv sind, wurde gezeigt, dass ERα ein nicht-geprimtes Substrat für GSK-3 darstellt.

Der in dieser Arbeit neu beschriebene GSK-3 / ERα – Signalweg zeigt, dass GSK-3 einen dualen Effekt auf die Funktion des ERα ausübt. In nicht-stimulierten Zellen wird ERα im Zytoplasma durch GSK-3 stabilisiert. Nach Stimulation mit E2 transloziert ERα in den Zellkern, wo er durch eine kernständige aktive GSK-3 phosphoryliert und aktiviert wird. Die Befunde erlauben die Schlussfolgerung, dass GSK-3 ein Bindeglied zwischen den schnellen nicht-genomischen, im Zytoplasma ablaufenden Prozessen und den im Zellkern ablaufenden genomischen Reaktionen des Liganden-aktivierten ERα darstellt.

Der ERα-Signalweg spielt eine kritische Rolle bei der Initiation und Progression von Brustkrebs. Deshalb stellt sich die Frage nach der Regulation von Funktion und Aktivität des ERα durch GSK-3 bei diesen Prozessen. Erste vorläufige Ergebnisse aus immunhisto-chemischen GSK-3β – Färbungen an Formalin-fixierten Gewebeschnitten menschlicher Mammakarzinome sprechen für eine vermehrte GSK-3β Expression in niedrig differenzierten (Grad 3) Tumoren im Vergleich zu gut bzw. mäßig differenzierten (Grad 1/2) Tumoren.

# I - Summary

Glycogen synthase kinase-3 (GSK-3), a serine/threonine kinase with docking properties, regulates numerous cellular processes. Two isoforms, GSK-3α and GSK-3β have been described. *In vivo*, GSK-3β is the major isoform and plays a key role in the regulation of transcription factors including steroid receptors. The aim of the present work, mainly performed on the estrogen receptor-α (ERα)-positive MCF-7 human breast cancer cell line, was to unravel the role of GSK-3 regarding ERα function.

After silencing of GSK-3α and/or GSK-3β isoforms using specific siRNA sequences, increased proteasomal degradation of ERα was observed. The use of the proteasome inhibitor MG132 restored ERα protein levels in GSK-3 silenced cells, showing that GSK-3 stabilizes ERα and protects it from proteasomal degradation. In another approach, specific silencing of the endogenous GSK-3β of MCF-7 cells using microRNA constructs was accompanied by down-regulation of ERα protein content. In these cells, ERα protein was rescued after overexpression of wild-type or kinase-inactive xenopus GSK-3β, which suggests that the docking properties of GSK-3 and not the kinase activity are important for ERα stabilization. Then, we found that 17β-estradiol (E2) -treatment resulted in rapid phosphorylation and consequent inactivation of cytoplasmic GSK-3. This GSK-3 phosphorylation may lead to ERα release from the GSK-3/ERα complex and ERα translocation into the nucleus, where it is phosphorylated at Ser-118 leading to its full activation. Upon E2 stimulation, treatment of the cells with the GSK-3 inhibitor LiCl resulted in a decrease of ERα phosphorylation at Ser-118. This decrease was confirmed upon silencing of GSK-3 in the nucleus and show that a nuclear active pool of GSK-3 is required for E2-induced phosphorylation of ERα at Ser-118. As a consequence, in GSK-3 silenced cells, E2-induced ERα transcriptional activity, studied by ERE-dependent luciferase reporter assays and by measuring transcription of the ERα-dependent target genes, pS2 and progesterone receptor, by quantitative real-time PCR, was significantly reduced. In GSK-3 silenced cells, neither Ser-118 phosphorylation nor luciferase activity was restored by use of MG132. Furthermore, overexpression of human GSK-3β wild-type and mutants inactive towards primed substrate of the kinase in MCF-7 cells stably transfected with an ERE-controlled luciferase reporter confirmed that GSK-3 triggers E2-induced ERα activation and suggests that ERα is a non-primed substrate of GSK-3 kinase. Taken together, this newly signalling pathway depicted a dual function of

GSK-3 regarding ERα, GSK-3 stabilises ERα in the cytoplasm of unstimulated cells and phosphorylates/activates the receptor in the nucleus upon E2 treatment. This permits the conclusion that GSK-3 represents a link between the rapid cytoplasmic non-genomic and the nuclear genomic actions of E2-liganded ERα.

Finally, ERα signalling pathway plays a crucial role in breast cancer initiation and progression. Therefore, the regulation of ERα function and activity by GSK-3 may have an impact on breast cancer progression. Preliminary data from GSK-3β immunostaining of formalin-fixed human tissue sections suggests a tendency toward an increase of GSK-3β expression in grade 3 tumors in comparison with grade 1/2 tumors.

# II - Introduction

## II - 1) Cancer overview

Cancer is one of the most frequent diseases and subject of extensive studies worldwide. Recently, some factors such as improvement of tumor diagnosis and longer human lifespan have increased the number of patients suffering from cancer. An early detection of cancer enhances the possible cure of the patient but the panel of therapies to heal cancer is not efficient enough. The aim of current cancer research is to better understand the aetiology and the progression of the tumor to find a strategy able to prevent, reduce, abolish and maybe eradicate cancer.

The growth of the different types of cells constituting each tissue of the human body is tightly regulated. In cancer, the homeostasis is disrupted. In other words, the rates of cellular proliferation and apoptosis are not anymore in balance. This un-controlled growth leads to the formation of a neoplasm or tumor. Benign tumors grow locally and can neither invade healthy surrounding tissues, nor metastasize in distant organs. On the contrary, malignant tumors can spread by invasion and metastasis. The term “cancer” applies only for malignant tumors. Uncontrolled growth that progresses toward limitless expansion is the hallmark characteristic shared by all the types of cancer (definition of the National Cancer Institute, http://www.cancer.gov).

Cancer classification was established according to the nature of the tissue from which the tumor has originated. Sarcomas are cancers arising from connective or supportive tissues of the body that are derived from mesenchymal cells such as bone, fat and muscle. Secondly, leukemias and lymphomas are cancers arising from hematopoietic cells of the bone marrow and from tissues of the body’s immune system, respectively. Finally, 90% of cancers are carcinomas, which arise from epithelial cells of the bladder, breast, colon, lung, prostate and skin. Among men, the most lethal cancer types in order of death numbers worldwide are lung, stomach, liver, colorectal, oesophagus and prostate carcinomas. Among women, the most lethal cancer types in order of death numbers worldwide are breast, lung, stomach, colorectal and cervical carcinomas (World Health Organisation, 2006, www.who.int). This dissertation will focus on breast cancer and it will describe and analyse biological mechanisms playing a crucial role in the initiation and progression of this harmful carcinoma.

## II - 2) Breast cancer

To understand breast cancer and more accurately the different types of breast cancer the structure and function of the breast was studied. Depending on the diagnosis, the stage of development and progression at the time point of detection and cellular origin, the breast cancer types will have different prognosis and the treatment strategy will be adapted to optimize the clinical outcome for the patient.

### II - 2.1) Anatomy of the breast

The breasts contain lobules and ducts, surrounded by fat tissue, which are responsive to many of the female hormones, such as estrogen and progesterone (**Fig. II - 1**). The lobules consist of tiny bulbs or glands (alveoli) that proliferate during pregnancy and are responsible for producing milk after delivery. The milk is then secreted into the ducts. These thin tubes start from the lobes (groups of lobules) and carry the milk to the nipple. Blood and lymph vessels drain the breast tissue. The lymph vessels lead to small, bean-shaped organs called lymph nodes. Groups of lymph nodes are located near the breast and in the axilla, or armpit. Some muscles that are important for the arms movements as well as for breathing are also found over the ribs under each breast.

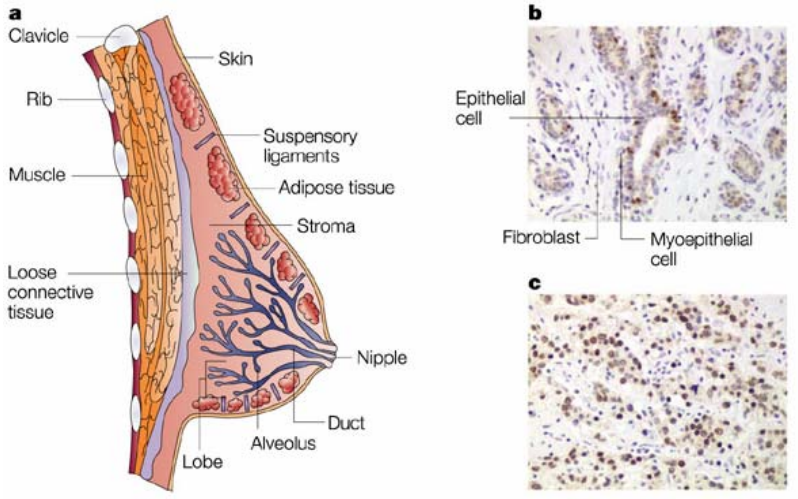


**Figure II - 1: Structure of normal and malignant breast tissue. a**) Anatomy of the human mammary gland. Each mammary gland contains 15-20 lobes, each lobe containing a series of branched ducts that drain into the nipple. **b**) The branches of the duct open into the alveoli that are formed of two cell layers, the luminal epithelial cells and the myoepithelial cells. The luminal cells are responsible for the milk production. The glandular ducts are embedded in stroma. **c**) This mesenchymal structure breaks down in breast cancer, resulting in an epithelial cell mass. **b** and **c** are immunostained using antibodies to the estrogen receptor α (ERα; brown stained nuclei), showing that only a small proportion of epithelial cells are ERα positive in the normal breast. The percentage of ERα-positive cells in breast cancer is highly variable and can cover less than 10 % to more than 70 % of the cancer cells (Ali and Coombes, 2002).

### II - 2.2) Types of breast cancer

Breast cancers may originate from either the lobules or the ducts and they are called lobular carcinoma and ductal carcinoma, respectively. “In situ” carcinomas are cancers that have not crossed beyond the lobular or ductal cell layers. They are opposed to invasive cancers that extend beyond the cell layer and basal membrane and metastasizing breast cancers, which spread outside the breast.

Ductal carcinomas are the most common type of breast cancer. Invasive ductal carcinoma (IDC) accounts for 85 % to 90 % of breast cancers (Ray and Mitra, 2003). This begins in the milk duct and it invades the surrounding fatty and connective tissues. It also has the possibility to metastasize. Unlike IDC, ductal carcinoma in situ (DCIS) is a non-invasive type of cancer located inside the milk ducts.

Lobular carcinomas are less common. They are also divided into LCIS (lobular carcinoma in situ) and ILC (invasive lobular carcinoma). LCIS are pre-cancerous neoplasias located in the lobules. ILC have the potential to invade surrounding tissue and to metastasize. They account for about 10 to 15% of breast cancer.

More rare type of breast cancers are sometimes diagnosed such as inflammatory breast cancer, medullary carcinoma, mucinous carcinoma, Paget’s disease of the nipple and phyllodes tumors.

### II - 2.3) Epidemiology and aetiology

Breast cancer is the most common cancer among women in Western countries. The American Cancer Society estimated that, in 2007, more 178480 new invasive cases were diagnosed among women in the United States. At this time, slightly over 2 million women living in the United States have been diagnosed with and treated for breast cancer. One woman in eight either has or will develop invasive breast cancer in her lifetime. This represents the highest rate of breast cancer among women in the world. Each year in the United States, about 40460 women and 450 men die due to breast cancer (American Cancer Society, 2007, http://www.cancer.org). In comparison, 47500 women were diagnosed among women in 2003 in Germany and 17173 women died from breast cancer (Robert Koch Institute, http://www.rki.de).

Concerning the aetiology of breast cancer, some risk factors are out of woman’s control, such as her age or family history. Indeed, the older a woman is, the greater is her risk to get breast cancer. Genetics and familial factors will increase even more the probability of cancer development. At the opposite, some risk factors are under a woman’s control and

concern her lifestyle. For example, drinking alcohol and smoking increase the rate of breast cancer. Some non-classified risks such as obesity, early menarche onset, late menopause and delayed first pregnancy or absence of pregnancy may also increase the incidence of breast cancer. Finally, breast cancer development and breast tissue homeostasis are under the influence of endogenous and exogenous hormonal agents. The number of mammary carcinoma cases rises dramatically for over-fifty years old women and corresponds to physiological hormonal modifications of estrogen and progesterone ratios due to the menopause. In addition, hormone replacement therapy (HRT) used as an exogenous postmenopausal treatment has been controversially shown by some epidemiological studies to increase breast cancer occurrence (National Cancer Institute, http://www.cancer.gov; Medlineplus, http://www.nlm.nih.gov/medlineplus).

### II - 2.4) Detection and diagnosis

Breast cancer cases are mostly discovered either by self-examination or by mammography imaging. The aim is to detect the cancer as early as possible to have better chance of recovery and disease-free survival.

Self-exam is the first recommended method to detect cancer. A woman should check her breasts monthly for possible changes. If a lump can be felt in the breast, the advice is to perform a clinical breast exam. Over the age of 40, this exam is recommended yearly since mammographs seem to be the most reliable method to detect breast cancer. This technique uses X-ray to detect alterations in woman's breasts. Deposits of calcium can also be detected. They are mostly benign, but a cluster of them may be an early sign of breast cancer. "False negative" and "false positive" findings can disturb the diagnosis. Therefore, biopsies can be performed for histopathological classification of the findings. These studies of fluid or tissue removed from the suspicious area will improve the diagnosis.

When breast cancer has been detected, further tests must help to identify the possible presence of metastasis. Radiological tests including bone scan and different computerized axial tomography (CAT) scans are performed to check whether the cancer has spread outside the breast, especially in the axillary lymph nodes.

### II - 2.5) Staging

The diagnosis of a cancer includes the classification of this cancer into a well defined stage to follow tumorigenesis and tumor progression more precisely and to find the best treatment strategy. The 'TNM' classification system is a standard system used to describe the

growth and the spreading rates of tumors (**Table II - 1**). 'TNM' stands for 'Tumor-Nodes-Metastasis' and respectively indicates the tumor's size (T), whether lymph nodes (N) in the area of the original tumor have become infiltrated by cancer cells and whether the cancer has spread to other organs (M or metastasis). Then, according to information from the 'TNM' classification, a patient's overall disease stage is determined.

| **Size of the Primary Tumor (T)** | **Definition** |
|---|---|
| **TX** | Primary tumor cannot be assessed. |
| **T0** | No evidence of primary tumor. |
| **Tis** | Pure carcinoma in situ: LCIS, DCIS or Paget disease of the nipple with no associated tumor mass. |
| **T1** | Tumor 2 cm or less in greatest dimension. |
| **T2** | Tumor more than 2 cm but not more than 5 cm in greatest dimension. |
| **T3** | Tumor more than 5 cm in greatest dimension. |
| **T4** | Tumor of any size growing into the chest wall or skin. |
| **Lymph Nodes (N)** | **Definition** |
| **NX** | Regional lymph nodes cannot be assessed. |
| **N0** | Cancer has not spread to regional lymph nodes. |
| **N1** | Cancer has spread to 1 to 3 lymph node(s) in the axilla |
| **N2** | Cancer has spread to 4 to 9 lymph nodes in the axilla. |
| **N3** | Cancer has spread to 10 or more lymph nodes in the axilla or also involves lymph nodes in other areas around the breast. |
| **Metastasis (M)** | **Definition** |
| **MX** | Presence of distant spread (metastasis) cannot be assessed. |
| **M0** | No distant spread. |
| **M1** | Spread to distant organs is present. |

**Table II - 1: TNM classification of breast cancer** (Singletary et al., 2002).

Breast cancer stages are usually expressed with Roman numbers from 0 to IV. A low number indicates an early stage of cancer. Stage 0 (Tis, N0, M0) is sometimes called non-invasive carcinoma or carcinoma in situ. Cells that look like cancer are located in either a breast lobule (LCIS) or a breast duct (DCIS) and these cells did not cross beyond the basement membrane to invade the surrounding tissues.

Stages I and II are considered early stages of breast cancer. In the stage I (T1, N0, M0), breast carcinoma become invasive and can be considered now onward as a cancer. The

primary tumor measures no more than 2 cm and has not spread outside of the breast. In the stage II, different types of breast cancer are classified. In stage IIa, the primary tumor can measure less than 2 cm and has spread nearby to the lymph nodes in the axilla (T0, N1, M0 or T1, N1, M0) or the primary tumor can also be bigger (2-5 cm in size) and may or may not have spread to the lymph nodes under the arm (T2, N0, M0). In stage IIb, the primary tumor is larger than 5 cm and has not spread outside the breast (T2, N1, M0 or T3, N0, M0).

Stages III and IV are considered advanced stages of breast cancer. In the stage IIIa (T0-2, N2, M0), the cancer has really taken hold in the lymph nodes. In stage IIIb (T4, N0-2, M0), the primary tumor has grown into the chest wall and/or skin. In stage IIIc (T0-4, N3, M0), tissues around the breast such as skin or chest wall may be invaded by tumor cells with spreading to 10 or more lymph nodes in the axilla; or to 1 or several lymph nodes under the clavicle (infraclavicular) or above the clavicle (supraclavicular); all these on the same side as the breast primary tumor. Stage IV (T0-4, N0-3, M1) represents metastatic stages of cancer. The cancer has spread beyond the breast, leading to secondary tumors in the bones, the lungs, the liver, the brain or elsewhere.

The percentage of patients who live at least 5 years after their cancer is diagnosed (also called 5-year survival rate) decline according to the breast cancer stages. This 5-year survival rate is 100 %, 100 %, 92 %, 81 %, 67 %, 54 % and 20 % for the stage 0, stage I, stage IIa, stage IIb, stage IIIa, stage IIIb and stage IV, respectively (American College of Surgeons National Cancer Data Base, http://www.facs.org/cancer/ncdb, 1998). The stage IIIc was not estimated since it was only recently described (system revised in 2002). Moreover, the survival rates should be better now with the improvement of treatment strategies.

### II - 2.6) Grading

Histological grades of patient's cancerous breast tumors are determined by pathologists to identify the type of tumor present, including cellular structure and function. The histological grading of breast cancer helps to determine prognosis for the patient and to orientate the medical treatment. The most common grading system used today is the Scarff-Bloom-Richardson (SBR) system (Scarff and Torloni, 1968; Bloom and Richardson, 1957). After breast biopsy, lumpectomy or mastectomy, a sample of breast tissue is examined under the microscope. To determine the cancer's grade (**Table II - 2**), three features are closely observed: the frequency of cell mitosis (rate of cell division), tubule formation (percentage of carcinoma composed of tubular structures) and nuclear polymorphism (change in size and uniformity of nuclei). To each of these features, a score ranging from 1 to 3 is assigned and

the final sum will range the breast cancer between 3 and 9. Grade 1 tumors are well differentiated with a sum of 3, 4 or 5. Grade 2 tumors, moderately differentiated, have a score of 6 or 7. Grade 3 tumors are poorly differentiated and reach 8 to 9. Some other features such as necrosis are also reported by pathologists when determining the tumor's grade.

| Grade | Description | Score | 5 years survival | 7 years survival |
|---|---|---|---|---|
| **Grade 1** (slower cell growth) | Well differentiated breast cells; cells generally appear normal and are not growing rapidly; cancer arranged in small tubules. | 3, 4, 5 | 95 % | 90 % |
| **Grade 2** | Moderately differentiated breast cells; have characteristics between Grade 1 and Grade 3. | 6, 7 | 75 % | 63 % |
| **Grade 3** (faster cell growth) | Poorly differentiated breast cells; cells do not appear normal and tend to grow and spread more aggressively. | 8, 9 | 50 % | 45 % |

**Table II - 2: Scarff-Bloom-Richardson (SBR) grade system and survival prognosis** (Scarff and Torloni, 1968; Bloom and Richardson, 1957).

### II - 2.7) Treatment strategies

Death rates from breast cancer have been declining due to earlier detection and improved treatment. The fewer tumor cells are present before the treatment, the higher is the probability of healing the cancer. According to the stage of breast cancer and for every patient, the most appropriate combination of the strategies described below must be chosen to reach the best efficiency of treatment and to minimise the side effects.

Most breast cancers are taken out surgically. The aim of a lumpectomy is to remove the cancerous lump completely and to perform biopsies of some axillary lymph nodes to check for possible spreading. Mastectomy can also be performed. This technique is a total or partial removal of the breast, along with most of the lymph nodes in the axilla. Surgery alone is only efficient if the cancer has been detected at an early stage, when it has not spread elsewhere in the body. To prevent recurrence of a cancer, the surgeon may recommend some therapies in addition, including radiation therapy, hormonal therapy and chemotherapy. Radiation therapy is often used after surgery to destroy any remaining cancer cells. The use of high-energy rays (X-rays) to kill cancer cells also causes severe DNA damages to the healthy cells.

Chemotherapy is mostly used to treat cancer that has spread to other organs of the body or to prevent cancer from spreading outside the breast. Chemotherapy consists of a combination of drugs given orally or by injection to kill cancer cells. The drugs can block cell proliferation, disrupt the cell cycle or induce cell apoptosis. The treatment efficiency and the side effects of the drug are proportional to the concentration of drug reaching the tumor and to the duration of the treatment.

Breast cancer is a typical hormone-dependent neoplasm. Estrogen is the key hormone involved in the development and progression of almost all mammary tumors. The estrogen receptors are the mediators of estrogenic actions in breast cancer. About 70 % of breast cancers are positive for estrogen receptors (Johnston and Dowsett, 2003). Hormone therapy is therefore based on either blocking the activation of estrogen receptor by estrogen or preventing estrogen synthesis (**Fig. II - 2**). Antiestrogens including selective estrogen receptor modulators (SERM), selective estrogen receptor destabilisators (SERD), aromatases inhibitors and releasing hormone analogs have been used after breast tumor removal by surgery. Tamoxifen was the first SERM discovered in 1967 by Harper and Walpole and inhibits the activation of estrogen receptor in a tissue selective manner. Tamoxifen is widely used today and is considered as the SERM of reference. However, critical side effects have been reported and recently, other SERMs such as raloxifene have reached clinical trial. Fulvestrant (faslodex) is a newly approved SERD, which eliminates estrogen receptor. Then, aromatase inhibitors (letrozole, anastrozole, exemestane) represent a promising way to treat breast cancer. Indeed, the aromatase enzyme converts androgens (androstenedione) to estrogen (estrone) and is the ultimate source of estrogen. Aromatase inhibitors are only efficient for postmenopausal women, whose estrogen production is in small amounts. Lowering the level of estrogen can be achieved for premenopausal women by ovarian ablation. Some luteinising hormone-releasing hormone (LHRH) or gonadotropin releasing hormone (GnRH) analogs such as goserelin or leuprolide can also be given to block ovaries making estrogen.

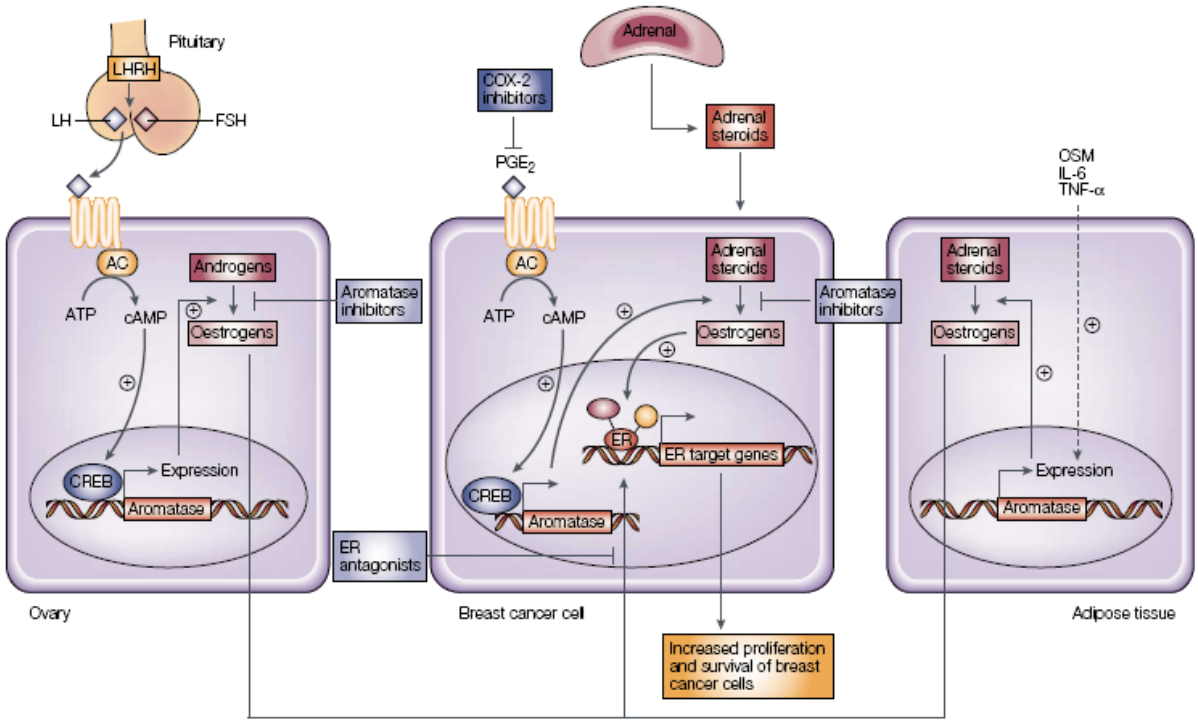


**Figure II - 2: Mechanism of action of therapeutic agents used in endocrine therapy.** Ovarian estrogen synthesis is regulated by the pituitary, which releases luteinising hormone-releasing hormone (LHRH). This, in turn, regulates the release of luteinising hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. In the ovary, both LH and FSH increase intracellular levels of cyclic AMP (cAMP). This activates the transcription factor cAMP-response-element-binding protein (CREB) and increases the expression of aromatase, which catalyses the conversion of ovarian androgens to estrogens. LHRH agonists first cause a release of stored LH and FSH and a subsequent LHRH-receptor downregulation that inhibits further LH and FSH release. In the normal breast, adipose tissue is a local source of estrogen synthesis. Factors that regulate aromatase expression, resulting in local estrogen synthesis, include interleukin-6 (IL-6), oncostatin M (OSM) and tumour necrosis factor-α (TNF-α). In breast cancer, the cancer cells themselves synthesize estrogens, and aromatase inhibitors are used to suppress local production of estrogens from adrenal steroids, as well as reducing ovarian estrogen synthesis. Moreover, locally produced prostaglandin E2 (PGE2) seems to be an important regulator of aromatase activity. PGE2 can activate prostaglandin E receptors on breast cancer cells, which increase cAMP levels and activate aromatase expression. Cyclooxygenase-2 (COX-2) antagonists, by blocking prostaglandin production, might inhibit this pathway. Finally, anti-estrogens inhibit estrogen action directly by binding to the estrogen receptor (ER). AC, adenylyl cyclase (Ali and Coombes, 2002).

In addition, progesterone receptors described in some breast cancer (Johnston and Dowsett, 2003) may be used as a potential target to treat this disease. However, progesterone receptor antagonists are still under pharmaceutical investigations for the treatment of breast cancer (Hoffmann and Sommer, 2005).

Finally, growth factors and growth factor receptors promoting tumor progression are potential therapeutic targets. In some breast cancer, the epidermal growth factor receptor ErbB2 (or HER2/neu) is overexpressed in the cancer cells and this correlates with reduced patient survival (Baselga, 2006). Monoclonal antibodies have been developed to decrease

proliferation of cancer cells overexpressing such growth stimulating receptors. Trastuzumab is the only validated example so far of humanised monoclonal antibodies. This antibody targets the extracellular region of the ErbB2 receptor and selectively inhibits the growth of cancer cells overexpressing this receptor. However, the impact of this monoclonal antibody treatment is limited since amplification of ErbB2 occurs in 25-30% of early-stage breast cancer (Bange *et al*., 2001) and resistance to the treatment rapidly arises.

## II - 3) Estrogen signalling and breast cancer

Hormones can be defined as chemical signals secreted by glands into the blood stream that act on distant tissues, usually in a regulatory fashion (Larsen *et al*., 2002). Hormones act on cells either via binding to extracellular receptors and second messenger activation or via intracellular receptors. Steroid hormones are lipophilic ligands that can diffuse freely trough the cytoplasmic membrane of the target cells and bind to intracellular receptors know as nuclear receptors. Indeed, hormone binding will phenotypically change the conformation of these receptors and allow them to cross the nuclear membrane and to enter the nucleus. In this cell compartment, the hormone-receptor complex will selectively bind to hormone response elements and promote the transcription of appropriate target genes.

Breast development and function are under the influence of hormones. Unsurprisingly, breast cancer is therefore a classical hormone-dependent neoplasm. The two hormones essential for mammary gland growth and development are estrogen and progesterone. The roles of these steroidal hormones and their nuclear receptors in breast tumorigenesis must be clarified but estradiol has an obligate role in the development of breast cancer (Anderson and Clarke, 2004). In particular, about 70 % of primary breast cancers in women are ERα positive and show estrogen-dependent growth. In the following, the synthesis, the mechanisms of action and the effects of estrogen will be especially described to further analyse its involvement in breast cancer.

### II - 3.1) Estrogen biosynthesis and function

Steroid hormones are derived from the cholesterol (**Fig. II - 3**). Tissues involved in steroid transformation receive cholesterol from the blood and can also synthesise this compound *in situ*. In these tissues, hydroxylases transform cholesterol into pregnenolone. The following step depends on the enzymatic endowment of each tissue and the steroids

synthesised are consequently different. In the adrenal glands, either $C_{21}$ steroids (aldosterone and cortisol) or $C_{19}$ steroids (dehydroepiandrosterone or DHEA) are produced. The ovaries and the placenta are enzymatically equipped with aromatases (a cytochrome P450 enzyme complex) and $C_{18}$ steroids (estrogens) can be additionally synthesised.

The naturally occurring estrogens are the estrone, the estriol and the estradiol. The most abundant and biologically most active circulating form of estrogen in the human body is the 17β-estradiol (E2). E2 was first discovered by Allen and Doisy in 1923. E2 is synthesised primarily in the ovaries. Ovarian synthesis of estrogen ceases after the menopause, but non-ovarian estrogen is still produced in the breast and adipose tissue for example (Ali and Coombes, 2002). To regulate their various biological functions, estrogens (i.e. E2) bind to estrogen receptors in the same cells where estrogen is produced (autocrine activation), in neighbouring cells (paracrine activation) or in cells of distant organs (endocrine activation).

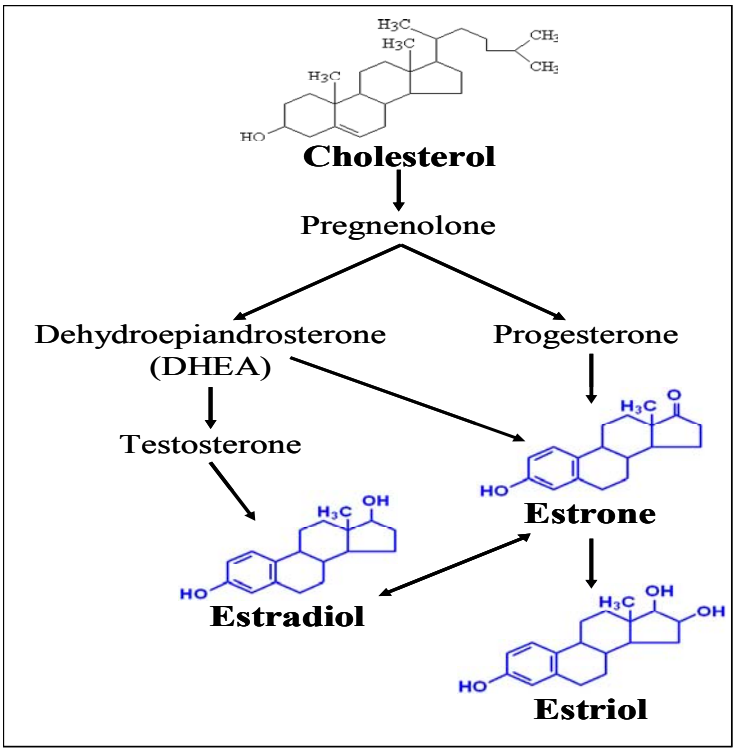


**Figure II - 3: Biosynthesis of estrogens from cholesterol.** The cholesterol comes from the triglycerides in diet and is transformed via enzymatic reactions into hormone precursors and finally active hormones such as the estrogens. Estradiol, estrone and estriol represent respectively 10%, 10% and 80% of the female estrogens within the whole human body.

## II - 3.2) Mechanism of estrogen receptors function and activity

### II - 3.2.1) Structure and tissue expression of estrogen receptors

Estrogen receptor was first discovered in 1962 by Jensen and Jacobson. Estrogen receptors (ER) are categorised into the class I of nuclear hormone receptor superfamily of proteins and function as transcription factors. Estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) have been identified. These two proteins come from distinct genes located on separated chromosomes (6q 25.1 and 14q 22-24, respectively; Menasce *et al*., 1993; Enmark *et al*., 1997). However, their structures are similar (**Fig. II - 4**). They consist of a variable amino-terminal A/B domain, which contains a ligand-independent activation function (AF-1); a C domain composed of two zinc fingers, which is required for DNA binding; a D domain or hinge region, which contains the nuclear localisation signal; an E domain, which contains a ligand-dependent activation function (AF-2); and a C-terminal or F domain with so far unclear function (Henry and Norman, 2003). AF-2 requires the binding of estrogen to be activated, whereas AF-1 activity is regulated by phosphorylation. These activation domains act independently and/or synergistically to regulate the transcriptional activity of target genes (Ali and Coombes, 2002).

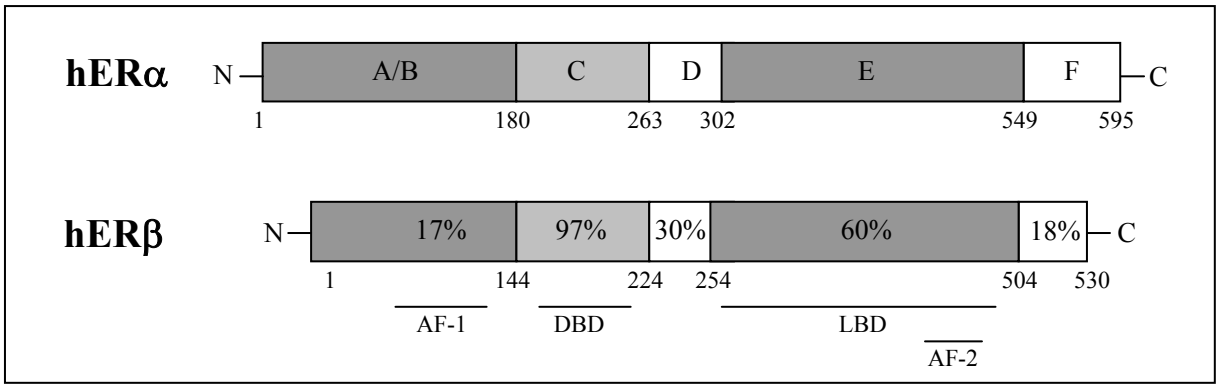


**Figure II - 4: Domain structure representation of human ERα and ERβ subtypes.** Domains (labelled A-F), amino acid sequence numbering, two activation functions (AF-1 and AF-2), and percentage of homology between the two receptor subtypes in different regions, including the DNA-binding domain (DBD) and ligand binding domain (LBD) are shown (adapted from Kong *et al*., 2003; Moggs and Orphanides, 2001).

The two subtypes of ER have a high degree of homology regarding their C domain. They differ essentially at their extremities in the A/B domain and in the F domain. Moreover, they show different patterns of tissue expression (Katzenellenbogen *et al.*, 2000). Tissue responsiveness to estrogenic compounds is believed to depend on the expression patterns of

ER subtypes and their coregulators (Moggs and Orphanides, 2001). ERβ exhibits the most limited expression pattern and is primarily detected in the ovary, prostate, testis, spleen, lung, hypothalamus and thymus (Couse *et al.*, 1997). At the opposite, ERα can be detected in almost all tissues. ERα is the predominant subtype expressed in the breast, uterus, cervix and vagina (Hall et al., 2001).

### II - 3.2.2) Cell signalling of estrogen receptors

Four signalling pathways were reported to mediate biological effects of estrogen receptors (**Fig. II - 5**). Three of these pathways include genomic effects of ER and the fourth pathway leads to non-genomic estrogenic activity (Hall *et al.*, 2001).

The first classical mechanism is a ligand-dependent activation of ER. Upon E2 stimulation, conformational modifications of estrogen receptors lead to increased phosphorylation of the receptors (Lannigan *et al.*, 2003; Likhite *et al.*, 2006). Regarding the ERα subtype, phosphorylation at serine residues was observed in a number of different cell types (Le Goff *et al.*, 1994). The protein kinases involved in these phosphorylations will be described in the part II - 4.3 of this introduction. Concerning ERβ, phosphorylation sites are still unclear. Nevertheless, phosphorylation seems to be a crucial step in the activation of ER signalling pathway for both receptor subtypes. It may lead to ER dimerization and help to the recruitment of co-activators (Likhite *et al.*, 2006). Homodimerization (α/α or β/β) or heterodimerization (α/β) can occur, but the respective contribution of each dimer is not well clarified so far. Overall, the dimers are able to recruit specific coactivators to become functionally active and to bind estrogen response elements (ERE) located in the promoter region of target genes. The consensus ERE is a 13 base pair (bp) palindromic sequence (GGTCAnnnTGACC) consisting of two 5-bp inverted repeats with a 3-bp spacer (Henry and Norman, 2003). Each ER molecule of the dimer will bind to a 5-bp repeat and together, they will regulate transcription of target genes.

Second, growth factors such as the epidermal growth factor (EGF) or insulin-like growth factor-1 (IGF-1) can activate ERα in a ligand-independent manner. Modification of the phosphorylation state of the ERα seems to be also an important mechanism in the activation of this second pathway. The mechanisms are not entirely clear but phosphorylation of ERα may be sufficient to activate dimerization of the receptor and to regulate the transcriptional activity of ERE-dependent target genes. The ligand-dependent and ligand-independent ERα signalling cascades are relatively similar but the panel of genes regulated by

these two pathways may be different. Even more, each growth factor implicated may have different effects on the regulation of gene expression.

Third, an ERE-independent genomic action of estrogen can take place. After E2 stimulation, the complex agonist/ERα can lead to gene regulation in the absence of direct DNA binding. Indeed, this complex may interact with other transcription factors such as specificity protein 1 (Sp-1) and activator protein-1 (AP-1) and activate their responsive elements.

Another mechanism of ERα activation includes non-genomic effects of estrogens. Cell-surface bound ERα seems to be directly linked to intracellular signal transduction proteins and several protein kinases may especially be implicated. However, the functional relevance of this E2 signalling pathway is still under debate.

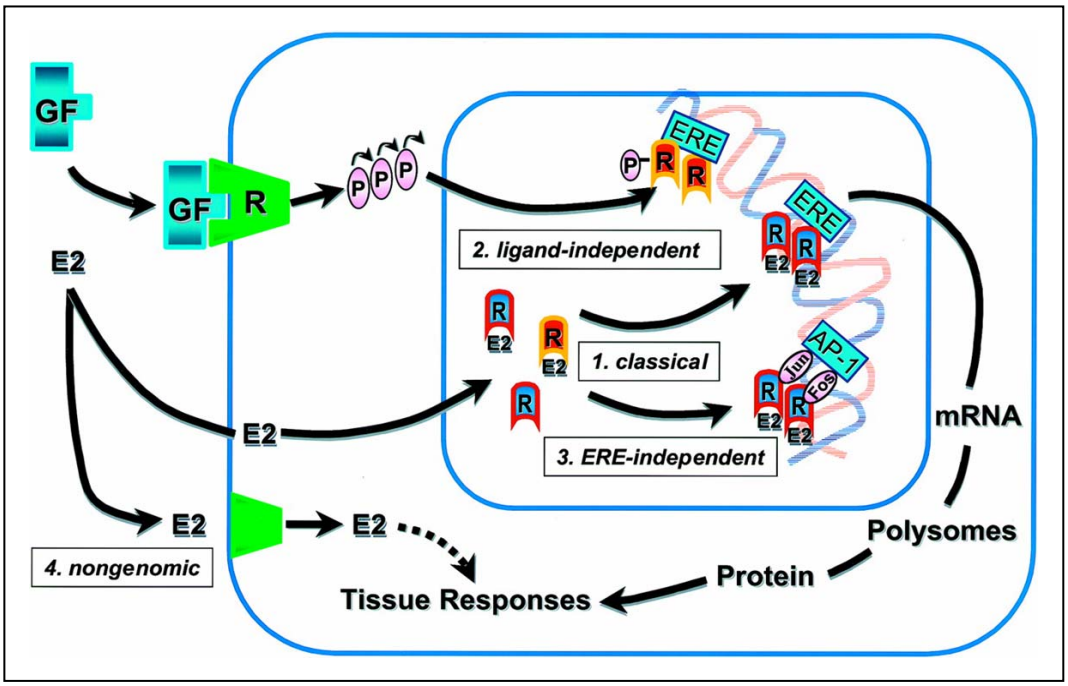


**Figure II - 5: The multifaceted mechanisms of estradiol and estrogen receptor signalling.** The biological effects of ER are mediated trough at least four signalling pathways. **1**) *classical ligand-dependent*, E2-ER complexes bind to ERE in target gene promoters leading to an up- or down-regulation of gene transcription and subsequent tissue responses. **2**) *ligand-independent*, growth factors (GF) or cyclic adenosine monophosphate (not shown) activate intracellular protein kinase pathways, leading to phosphorylation (P) of ER, receptor dimerization and binding to ERE-containing promoters of target genes. **3**) *ERE-independent*, E2-ER complexes alter transcription of genes containing alternative response elements such as AP-1 binding sites through association with other DNA-bound transcription factors (Fos/Jun), which tether the activated ER to DNA, resulting in an up- or down-regulation of gene expression. **4**) Cell-surface (*non-genomic*) signalling, E2 activates a putative membrane-associated binding site, possibly a form of ER linked to intracellular signal transduction pathways that generate rapid tissue responses (Hall *et al.*, 2001).

### I - 3.2.3) Biological function of estrogen receptors

According to the expression patterns of ER subtypes within a tissue and to the ER signalling pathways activated, estrogen receptors have multiple actions in reproductive tissues (such as breast, uterus and ovary) and in many non-reproductive tissues including bone, the central nervous system (CNS) and the cardiovascular system (Stossi *et al.*, 2004). The different phenotypes observed for ERα, ERβ and double ER null mice gave us valuable information on the impact of these receptors for the development and functionality of different tissues (Couse and Korach, 1999; **Table II - 3**) and notably showed that ERα was the main receptor responsible for these phenotypes. As an example, the mammary gland was immature and insensitive to estradiol in ERα null mice and no further alterations were observed in the double ER null mice.

| Tissue/system | αERKO | βERKO | α/βERKO |
|---|---|---|---|
| Fertility | Females and males infertile | Females subfertile, decreased litter size; males fertile | Both sexes infertile (resembles αERKO phenotype) |
| Female reproductive system | | | |
| Uterus | Hypoplastic uterus; no uterotropic response to estradiol; no implantation | Normal responses to estradiol; can support normal pregnancies | Resembles αERKO |
| Ovary | No ovulation; immature follicles and appearance of hemorrhagic cysts at puberty; "trapped follicle" phenotype after superovulation; elevated levels of estrogen and testosterone | Normal appearance; reduced ovulation | Granulosa cells undergo sex reversal, become Sertoli-like cells |
| Male reproductive system | | | |
| Testes | Normal development; testes decrease in weight with age; fluid resorption in efferent ducts decreases with age; sperm have poor motility, cannot fertilize oocytes *in vitro* | Normal | Resembles αERKO phenotype |
| Mammary gland | Immature; only a ductal rudiment present (development not stimulated by estradiol) | Normal development; normal lactation | Immature; resembles αERKO phenotype |
| Bone | Female: decreased bone diameter; male: decreased density; both sexes are shorter than wild type | Normal in males; increase in bone density in females | Both sexes shorter |
| Cardiovascular | Estradiol protection normal; decreased basal NO activity | Normal estradiol protection | Estradiol does not protect against increases in vascular medial area, but still protects against proliferation after injury |
| Gonadotropin/ hormone levels | Females and males: elevated levels of LH, estradiol, and testosterone; females: reduced prolactin levels | Normal | Elevated LH |
| Mating behavior | Decreased aggression; deficient mating behaviors | Normal sexual behavior | Males display no mounting behavior |

**Table II - 3: Phenotypes of ER knockout mouse models** (Henry and Norman, 2003).

### II - 3.3) Estrogenic activity and breast tumorigenesis

Estrogen regulates genes involved in the differentiation and proliferation of normal breast epithelial cells. Unsurprisingly, most breast cancers (about 95 %) are initially hormone-dependent, where the hormone estradiol plays a crucial role in their development (Pasqualini, 2004). Cumulative exposure of the breast epithelium to estrogen was particularly depicted as a risk factor associated with initiation and/or promotion of breast cancer. Two current hypotheses may explain the involvement of estrogen in breast cancer. In the first, binding of estrogen to its cellular receptor stimulates proliferation of mammary cells, increasing cell division and enhancing replication errors. In the second, estrogen metabolism leads to the production of genotoxic by-products that could directly damage DNA. Both of these

hypotheses may imply that estrogen induces mutations leading to the disruption of normal cellular processes such as cellular proliferation, apoptosis and DNA repair (Deroo and Korach, 2006). Breast cancer is therefore considered as the outcome of a complex and not fully understood interplay amongst genetic and hormonal factors (Ray and Mitra, 2003).

As described previously for the development of the mammary gland, ERα subtype is also the primary mediator of estrogenic actions in breast cancer. The estrogenic regulation of gene expression is under the influence of this receptor subtype. Moreover, several sequence variations or single-nucleotide polymorphisms (SNPs) in the ERα have been identified and associated with either increased or decreased risk of breast cancer. However, the hereditary contribution of SNPs for the development of breast cancer must be further investigated. Finally, alteration of the receptor expression was suggested to be the major cause of cancer progression and hormone-therapy resistance (Deroo and Korach, 2006).

### II - 3.3.1) Regulation of gene expression upon estrogen stimulation

In 2003, Frasor *et al.* performed gene expression profiling studies in estrogen receptor positive MCF-7 human breast cancer cells after E2 treatment for different time courses. For the genes regulated by estradiol, they observed that the majority of them (70 %) were downregulated. These include transcriptional repressors as well as anti-proliferative and proapoptotic genes. At the opposite, up-regulation was observed for positive proliferation regulator genes, for genes involved in cell cycle progression and for genes leading to the production of proteases playing a role in tumor invasiveness. In 2004, Terasaka *et al.* confirmed that estrogen target genes include primarily growth-associated genes and tumor-associated genes. They also showed that other genes such as ion-associated genes or genes coding for structural proteins may be regulated by E2.

Overall, estrogen mainly regulates genes resulting in growth and proliferation of breast cancer cells. Therefore, estrogens are considered as powerful enhancers of tumor progression.

### II - 3.3.2) Estrogen receptor status in breast cancer

The clinical outcome of breast cancer depends on ER and especially ERα status of the tumor. Allred et al. (2004) correlated the levels of ERα expression of invasive breast cancer assessed by immunohistochemistry with the clinical outcome of the patients (**Fig. II - 6**). They concluded that even if ERα was expressed in only few cells of the tumor, the clinical response to hormonal therapy was far better than in ERα negative tumors. Overall, estrogen-

dependent tumors have the tendency to grow faster than estrogen-independent tumors but the improvement of hormonal therapy may consider estrogen-dependent tumor easier to eradicate. Moreover, Allred *et al*. (2004) also showed that only a slight gradient of hormonal therapy efficiency was observed with better hormonal therapy response for tumors with high number of cells expressing ERα.

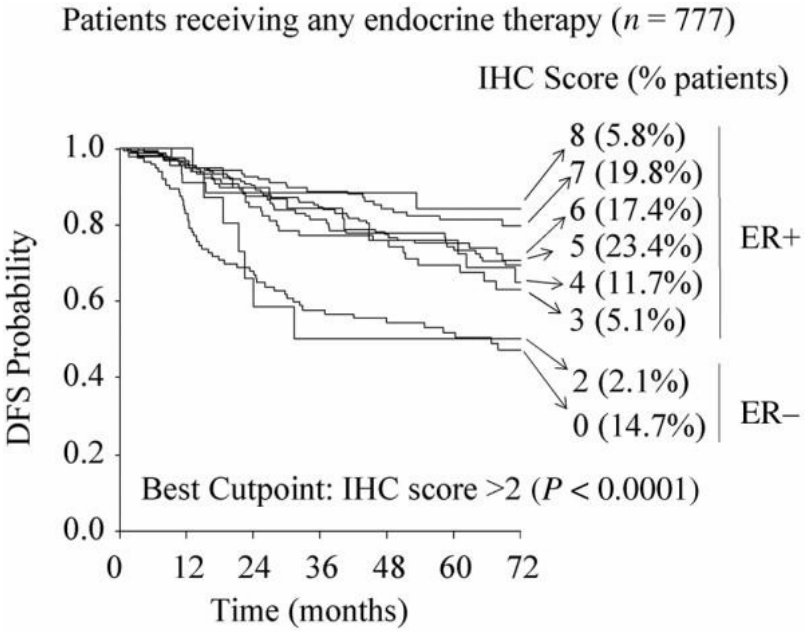


**Figure II - 6: Kaplan-Meyer curves illustrating relationship between ERα (ER) status (immunohistochemistry [IHC] scores) and clinical outcome (disease-free survival [DFS]).** Invasive breast cancer of patients (n=777) treated with adjuvant tamoxifen therapy were stained by IHC. IHC score equal to 0 and 2 corresponds to none and < 1 % ERα-positive (ER+) cells, respectively and were considered as ERα-negative (ER-) tumors. An IHC score of 3 represents tumors with 1-10 % of ER-positive cells and tumors with IHC score from 4 to 8 proportionally corresponds to > 10 % to 100 % ER-positive cells (Allred *et al.*, 2004).

## II - 4) Estrogen signalling-related protein kinases as key players of breast tumorigenesis

The protein kinase superfamily is one of the largest families in the human genome and regroups more than 518 members (Johnson and Hunter, 2005). Protein phosphorylation regulates important intracellular mechanisms and especially modification of proteins involved in the transmission of signals within the cell (signal transduction) such as ion transport, cellular proliferation and hormone responses. Therefore, protein kinases are very attractive targets for therapeutic interventions in many diseases such as cancer, diabetes, inflammation

and arthritis. In this regard, protein kinases represent as much as 30 % of all protein targets under investigation by pharmaceutical companies (Cohen, 2002).

As previously described, the regulation of estrogen signalling via ERα is under the influence of protein kinases and this signalling pathway is of major importance in breast cancer. Therefore, research investigation currently focuses on the specific protein kinases involved in this signalling cascade. The aim is to find target protein kinases of interest for the future development of protein kinase inhibitors to treat this hormone-dependent cancer.

### II - 4.1) The protein kinase superfamily

Protein kinases belong to a huge superfamily. In 1991, from catalytic domain alignment, Hanks and Quinn provided a general classification of protein kinases. This classification is still used. The protein kinases superfamily was divided into five protein kinase groups: AGC, CaMK, GMCC, PTK and OPK. Their names come from the proteins that belong to each of them. The AGC group includes protein kinases A, G and C, respectively regulated by cAMP, cGMP and diacylglycerol (DAG). The CaMK group corresponds to protein kinases regulated by calcium/calmodulin (CaM). The GMCC group includes glycogen synthase kinases (GSK) and especially glycogen synthase kinase-3β, mitogen-activated protein kinases (MAPK), cyclin-dependent kinases (Cdk) and the carboxy-terminal domain (CTD) kinases. The PTK group is composed of protein tyrosine kinases (PTK). The OPK or other protein kinases summarize some protein kinases such as MAPK/ERK kinase (MEK) and c-Jun N-terminal kinase (JNK) (Henry and Norman, 2003).

Protein kinases modify other proteins by chemically adding phosphate groups to them. A protein kinase transfers the γ-phosphate group from adenosine triphosphate (ATP) covalently to a hydroxyl group of a protein substrate (Henry and Norman, 2003). Most protein kinases act on both serine and threonine residues. Others act on tyrosine residues and some called dual specificity protein kinases act on all three. Therefore, protein kinases can be classified more easily according to the nature of the residue they prefer to phosphorylate. The serine/threonine protein kinase family include the AGC, CaMK and GMCC groups and some protein kinases from the OPK group. PTK constitute a separate family and the last family is composed of dual-specificity protein kinases.

The transfer of the phosphate group may functionally alter the target protein by changing its enzymatic activity, its cellular location or its association with other proteins. Protein kinases regulate the majority of cellular pathways such as cell growth, cell motility and cell death among others. Therefore, their activities are tightly regulated. Regarding the

protein kinase of interest, its regulation can be achieved through phosphorylation by other protein kinases (protein kinase cascade), by autophosphorylation, by binding of activators or inhibitors, or by controlling its location in the cell relatively to its substrates.

### II - 4.2) Protein kinases related to breast cancer

Thirty-two known cytoplasmic PTK have been determined so far and about half of them have been implicated convincingly in human cancer (Blume-Jensen and Tony Hunter, 2001). These PTK are involved in the signal transduction of receptor tyrosine protein kinases (RTK) playing a role in tumorigenesis, such as epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR) and fibroblast growth factor receptor (FGFR). In particular, for mammary carcinomas, overexpression of RTK including the ErbB2 member of the EGFR family and the FGFR4 have been reported (Koziczak and Hynes, 2004). This implies hyperactivation of the related PTK in these breast cancers.

Downstream of RTK are numerous cytoplasmic protein-serine/threonine kinases that have been linked to cancer. For example, MAPK signalling pathway has been involved in cell proliferation and cancer progression but no frequent recurring mutations have been identified in human mammary malignancies. In contrast, mutations of the PI3K/Akt and the mTOR/S6 kinase signalling pathways are involved in mammary tumorigenesis (Blume-Jensen and Tony Hunter, 2001). The most common genetic aberration in breast cancer is somatic mutation of the PIK3CA gene encoding for the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K). 26 % of primary breast tumors and 28 % of breast cancer cell lines displayed mutations in the PIK3CA gene (Bachman *et al*., 2004, Saal *et al*., 2005). Mutations in the p110α catalytic subunit of PI3K may result in activation of the PI3K signalling pathway and may contribute to mammary tumor progression (Dillon *et al*., 2007). Indeed, the PI3K pathway activates processes that fulfil hallmarks of cancer such as evading apoptosis, limitless replicative potential, insensitivity to anti-growth signals, sustained angiogenesis and tissue invasion and metastasis (Dillon *et al*., 2007). In addition, PKB/Akt, a downstream effector of PI3K, plays a role in breast cancer cell growth via the activation of mTOR signalling pathway (Li *et al*., 2004). Therefore, some of the transforming cell-growth and cell-cycle-promoting effects of PI3K are mediated through the mTOR pathway.

However, the overwhelming majority of protein kinases remain largely uncharacterized. Their relevance concerning breast cancer is under investigation and their role in the estrogen receptor signalling pathway must be specified.

### II - 4.3) Phosphorylation sites and protein kinases involved in ERα signal transduction

Lannigan *et al.* (2003) suggest that ERα becomes predominantly phosphorylated at Ser-118 and to a lesser extent at Ser-104 and Ser-106 in response to estradiol (**Fig. II - 7a**). Upon E2 stimulation, phosphorylation at Ser residues and particularly at Ser-118 in the AF1 domain appears to influence the recruitment of coactivators, resulting in enhanced ERα-mediated transcription (Likhite *et al*., 2006). Indeed, ERα mutated to Ala at Ser-104, Ser-106 and Ser-118 showed decreased estrogen-induced transcriptional activity in comparison with wild type ERα (Lannigan et al., 2003). However, the results were controversial with respect to the amino acid the Ser-118 residue was mutated to. ERα in which Ser-118 was mutated to Ala showed diminished transcriptional activity in response to E2 whereas ERα mutation at Ser-118 by an acidic residue could enhance the transcriptional response (Valley et al., 2005). While these phosphorylations are well established, the protein kinases involved are controversial. The protein kinases responsible for Ser-104/Ser-106 phosphorylation may be different from the one that phosphorylates Ser-118. Additionally, more than one protein kinase may phosphorylate a certain serine residue. In response to estradiol, cyclin A-cyclin dependent kinase 2 (Cdk2) complex phosphorylated Ser-104 and Ser-106, but it did not affect Ser-118 (Rogatsky *et al.*, 1999). According to Chen et al. (2000), Ser-118 may be phosphorylated by TFIIH cyclin-dependent kinase in response to estradiol. Additionally, activated MAPK also phosphorylated Ser-118, but not after E2 treatment (Joel et al., 1998).

Some other ERα serine residues such as Ser-167 and Ser-236 may also be phosphorylated by protein kinases (**Fig. II - 7b**). In particular, Ser-167 was described as a major site of phosphorylation in response to estradiol binding according to Arnold *et al.* (1994) but this observation was not confirmed by Le Goff *et al.* (1994) and Clark *et al.* (2001). Moreover, protein kinases such as Akt and ribosomal S6 kinase (Rsk) have been shown to phosphorylate Ser-167 residue *in vitro* and *in vivo* (Lannigan *et al.*, 2003). However, phosphorylation at Ser-167 by these protein kinases has not been reported in response to E2 suggesting that Ser-167 phosphorylation may have a role in ligand-independent ERα activation. Similarly, Ser-236 phosphorylation, hypothetically by PKA, may lead to ligand-independent dimerization of the receptor.

Finally, Tyr-537 phosphorylation of ERα may also occur and may be involved in the regulation of ERα sensitivity to E2 (Ali and Coombes, 2002). The protein kinases involved in this phosphorylation are unknown so far.

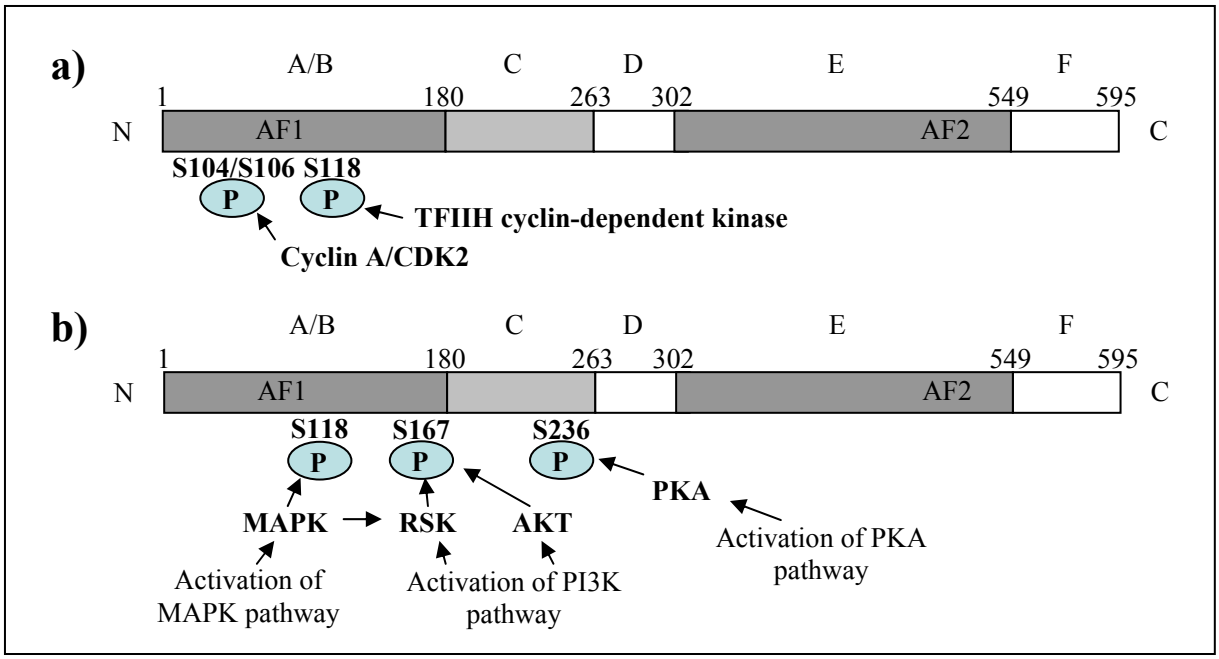


**Figure II - 7: ERα serine phosphorylation sites.** Phosphorylation of ERα at Ser residues is enhanced (**a**) in response to estradiol binding and (**b**) in response to second messengers leading to ligand-independent activation of the receptor. The serine phosphorylation sites and the protein kinases suggested to be responsible for these phosphorylations are shown (Lannigan *et al.*, 2003).

Moreover, rapid biological effects of E2 have been observed in the bone, breast, vasculature, and nervous system (Hall *et al.*, 2001). These nongenomic effects of estrogens may be mediated through cell-surface associated ERα and downstream activation of intracellular signal transduction proteins such as protein kinases. ERα localisation at the membrane is still controversial but Song et al. (2004) suggested that Shc and IGFR serve as key elements in the translocation of ERα to the cell membrane and in the facilitation of ERα-mediated rapid E2 action. In human MCF-7 breast cancer cells, estrogens via binding to the membrane-bound ER may rapidly activate several protein kinases, i.e., MAPK, PI3K, PKA and PKC. This rapid signal transduction cascades may be relevant for breast cancer cell proliferation in response to E2 (Vasudevan and Pfaff, 2007).

### II - 4.4) A putative role for the glycogen synthase kinase-3β (GSK-3β)

Recently, the serine/threonine protein kinase glycogen synthase kinase 3 (GSK-3) was described as a key regulator of many transcription factors. Some findings also postulate a possible effect of GSK-3 on the activity of steroid hormone receptors including ERα. The next parts will focus on GSK-3 kinase function and activity with emphasis on the possible role of GSK-3 kinase regarding the ERα signalling pathway.

Two highly homologous forms of mammalian GSK-3, GSK-3α and GSK-3β, have been described (Woodgett, 1990). The two isoforms are encoded by different genes and share nearly identical sequences (97% sequence similarity) in their protein kinase domain (Jopes and Johnson, 2004), while these isoforms have significant sequence differences outside from this region. The isoforms show similar substrate specificity but the isoform-specific functions are still unclear. The disruption of the GSK-3β gene in mice results in embryonic lethality, indicating that GSK-3α cannot completely compensate the loss of GSK-3β, whereas GSK-3β can overcome the loss of GSK-3α (Hoeflich *et al.*, 2000). Therefore, GSK-3β seems to be more essential. GSK-3β phosphorylates and thereby regulates the functions of many metabolic, signalling and structural proteins (Grimes and Jope, 2001).

### II - 4.4.1) GSK-3β substrates

GSK-3β has unique substrate specificity. Most GSK-3β substrates require a priming phosphate at *n* + 4 (where *n* is the site of phosphorylation) to be in turn phosphorylated by GSK-3β. For example, glycogen synthase (GS) must be phosphorylated at Ser-656 by casein kinase 2 (CK2) to allow GSK-3β phosphorylation at Ser-652, which in turn allows phosphorylation at Ser-648 and so on until five serine residues have become phosphorylated. Similarly, eukaryotic initiation factor-2B (eIF2B) might be phosphorylated by dual-specificity tyrosine-phosphorylated and regulated kinase (DYRK) at Ser-539 prior to GSK-3β phosphorylation at Ser-535 (**Fig. II - 8**).

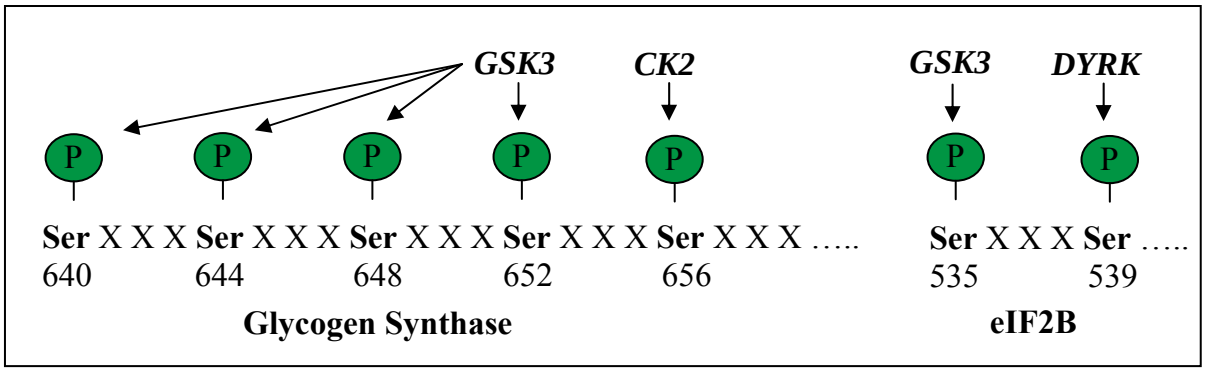


**Figure II - 8: The unique substrate specificity of GSK-3β.** Most substrates of GSK-3β must be phosphorylated by another protein kinase at a serine or a threonine residue located four residues carboxy-terminal to the site of GSK-3β phosphorylation. Schematic demonstration of the phosphorylation schedule of glycogen synthase and eukaryotic initiation factor-2B (eIF2B) proteins (adapted from Cohen and Frame, 2001).

However, other proteins such as axin and β-catenin, which are part of the Wnt pathway (Cohen and Frame, 2001), seem to be unprimed substrates of GSK-3β. Axin might bind to a distinct site of GSK-3β. Then, axin phosphorylation site might be directed to the active protein kinase site of GSK-3β, where axin is phosphorylated without primed phosphorylation by another kinase. A similar mechanism might also trigger the recruitment and phosphorylation of β-catenin without any priming phosphate (**Fig. II - 9**). GSK-3 inhibitors developed so far were ATP-competitive and did affect every substrates of GSK-3 regardless of their 'primed' or 'unprimed' properties. The difference between primed and unprimed substrates may help to develop compounds that target specifically the site that binds the 'priming phosphate' of substrates. Therefore, phosphorylation and activation of proteins that mediates insulin signalling (GS, eIF2B) may be inhibited without affecting 'unprimed' proteins such as members of the Wnt signalling pathway which are known for their involvement in oncogenesis (Cohen and Frame, 2001).

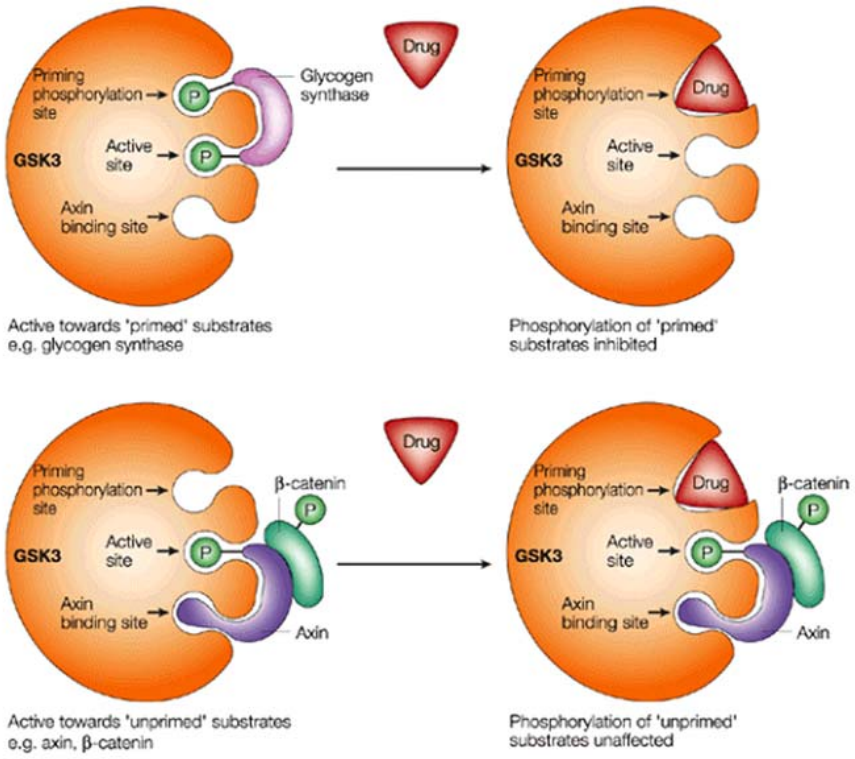


**Figure II - 9: GSK-3β substrates and their relevance for drug development.** Drugs that bind to the 'priming phosphorylation site' might mimic insulin signalling without triggering the Wnt signalling pathway (Cohen and Frame, 2001).

In conclusion, GSK-3β phosphorylates diverse substrates including metabolic and signalling proteins, structural proteins and transcription factors (**Table II - 4**). According to

Grimes and Jope (2001), one of the most important roles of GSK-3β is the regulation by phosphorylation of numerous transcription factors and thereby the control of the expression of the respective target genes. These transcription factors regulate genes involved in cell growth, cell proliferation and cell death such as AP-1. Others such as Myc and CCAAT/enhancer binding protein α (C/EBPα) contribute to the regulation of cellular differentiation. Some including nuclear factor kappa B (NFκB), nuclear factor of activating T cells (NFAT) and heat shock factor-1 (HSF-1) are involved in the immune system. Finally, CREB seems to be regulated by GSK-3β. This transcription factor plays a critical role in formation of long-term memory, maintenance of synaptic plasticity and apoptosis.

| Metabolic and signalling proteins | Structural proteins | Transcription factors |
|---|---|---|
| - AcetylCoA carboxylase<br>- Amyloid precursor protein (APP)<br>- Adenomatous polyposis coli (APC)<br>- ATP-citrate lyase<br>- Axin<br>- cAMP-dependent protein kinase<br>- Cyclin D1<br>- Cyclin E<br>- Eukaryotic initiation factor-2B (eIF2B)<br>- Glycogen synthase (GS)<br>- Insulin receptor substrate-1 (IRS-1)<br>- Myelin basic protein<br>- Nerve growth factor (NGF) receptor<br>- Protein phosphatase 1<br>- Protein phosphatase inhibitor-2<br>- Pyruvate dehydrogenase | - Dynamin-like protein<br>- Microtubule associated protein 1B (MAP1B)<br>- Microtubule associated protein 2 (MAP2)<br>- Neural cell-adhesion protein (NCAM)<br>- Neurofilaments<br>- Ninein<br>- Tau<br>- Telokin | - Activator protein-1 (AP-1)<br>- β-catenin<br>- CCAAT/enhancer binding protein (C/EBPα)<br>- Cyclic AMP response element binding protein (CREB)<br>- Glucocorticoid receptor (rat)<br>- Heat shock factor-1 (HSF-1)<br>- Myc<br>- Nuclear factor of activated T cells (NFAT)<br>- Nuclear factor kappa B (NFκB)<br>- Notch<br>- p53<br>- T-cell factor (TCF)<br>- Androgen Receptor<br>- ERα |

**Table II - 4: Mammalian protein putatively phosphorylated by GSK-3β** (updated from Grimes and Jope, 2001; Jope and Johnson, 2004).

### II - 4.4.2) GSK-3β regulation in a variety of intracellular pathways

Since GSK-3β has a predominant role in the control of several intracellular pathways, its activity needs to be carefully regulated. GSK-3β is one of the few protein kinases, which is inactivated by phosphorylation. In fact, serine phosphorylation inhibits GSK-3β activity, whereas tyrosine phosphorylation stimulates and increases its activity. Additionally, protein complex formation and intracellular localisation are other ways to regulate this enzyme.

Originally, GSK-3β was named for its ability to phosphorylate and thereby to inactivate the glycogen synthase (GS), which is a key regulator of glycogen metabolism (Grimes and Jope, 2001). Glycogen and glucose metabolism are regulated by insulin and the role of insulin is to decrease the level of blood glucose. Insulin binding to its cellular receptor triggers the activation of protein kinase B, which phosphorylates GSK-3β at Ser-9 residue and inhibits the activity of this enzyme (Cross *et al.*, 1995; **Fig. II - 10**). Indeed, the inactivation of GSK-3β promotes dephosphorylation of glycogen synthase at the sites phosphorylated by GSK-3β and contributes to the stimulation of glycogen synthesis (Frame and Cohen, 2001). By this way, insulin also stimulates the dephosphorylation and activation of eukaryotic initiation factor 2B (eIF2B), which is phosphorylated and inhibited by GSK-3β (Wang *et al.*, 2002).

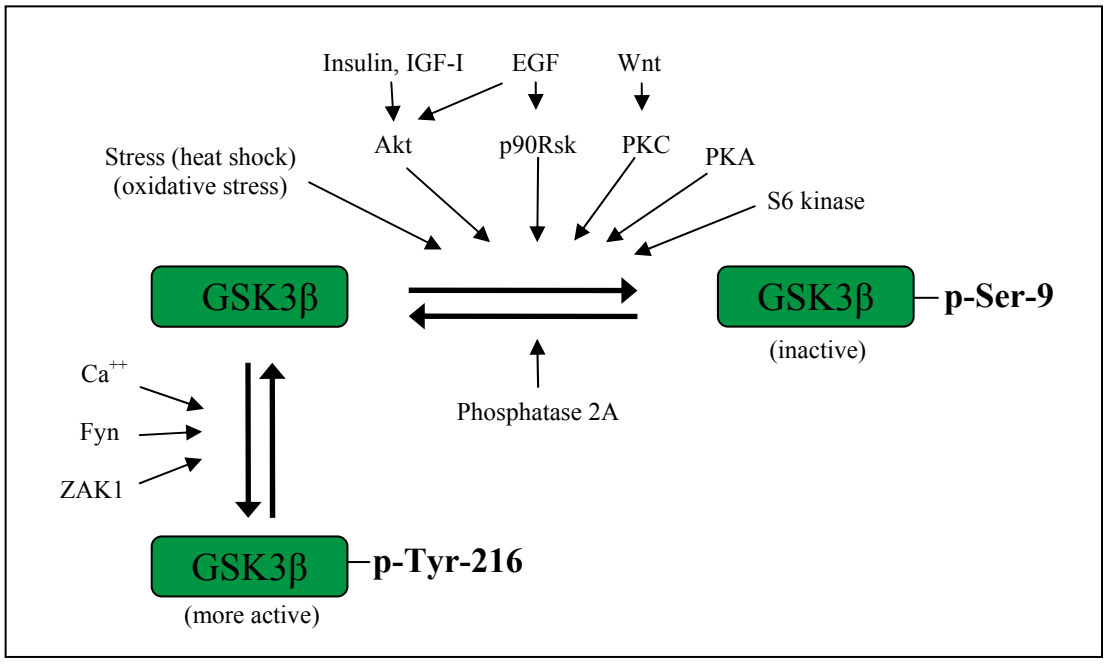


**Figure II - 10: Phosphorylation-dependent regulation of GSK-3β.** Some of the signals proposed to mediate activation of GSK-3β via phosphorylation of Tyr-216 (p-Tyr-216) and the inhibition of GSK-3β via phosphorylation of Ser-9 (p-Ser-9) are depicted (adapted from Grimes and Jopes, 2001).

Moreover, other proteins such as MAPKAP kinase-1 (p90Rsk), mammalian target of rapamycin (mTOR), PKA and PKC phosphorylate GSK-3β at Ser-9 residue (**Fig. II - 10**). These enzymes belong to growth factor and hormone-stimulated signal transduction pathways and stringently control GSK-3β.

Phosphorylation of GSK-3β at Tyr-216 increases the enzyme's activity. In 1994, Wang *et al.* suggested an autophosphorylation of this site. More recently, Kim *et al.* (1999) reported that ZAK1 kinase may increase GSK-3β activity via Tyr-216 phosphorylation in Dictyostelium. Lesort *et al.* (1999) suggest that Fyn, a member of the Src tyrosine protein kinase family, may also directly phosphorylate Tyr-216 and thereby activate GSK-3β. Additionally, increase of intracellular calcium and several proapoptotic stimuli were described to induce Tyr-216 phosphorylation and increase GSK-3β activity, but the protein kinases involved have not yet been identified.

GSK-3β activity can also be regulated by protein complex formation in the Wnt signalling pathway (**Fig. II - 11**). This mechanism was first described in xenopus and then extended to drosophila and mammals. In all these organisms, this pathway specifies cell fate, cell proliferation and cell differentiation (Frame and Cohen, 2001). Similar complex formation is observed in the Wnt signalling pathway between these organisms. The differences concern only the names of the proteins involved in this complex. Protein nomenclature in mammals will be used for the following description of the different components of this complex. In the absence of secreted glycoproteins known as Wnt, GSK-3β is active in the complex and phosphorylates adenomatous polyposis coli (APC), axin and β-catenin. The aim of APC phosphorylation is unclear, but it may enhance interaction between GSK-3β and β-catenin. Axin is stabilised by phosphorylation and β-catenin phosphorylation targets it for ubiquitination and subsequent proteolytic destruction. After the binding of Wnt to their receptors, the proteins dishevelled (DVL) together with frequently rearranged in advanced T-cell lymphomas (FRAT) bind to GSK-3β and inhibit its activity. Therefore, APC, axin and β-catenin will be dephosphorylated and released from the complex. Then, β-catenin translocates and accumulates in the nucleus, where it binds to members of the T-cell factor (TCF) family of transcription factors (also called LEF or lymphoid-enhancer factor). This stimulates the transcription of genes required for embryogenesis during developmental stage and the transcription of genes especially involved in tissue proliferation such as mammary development during lactation in the adult stage (Hatsell *et al*., 2003; Hens and Wysolmerski, 2005).

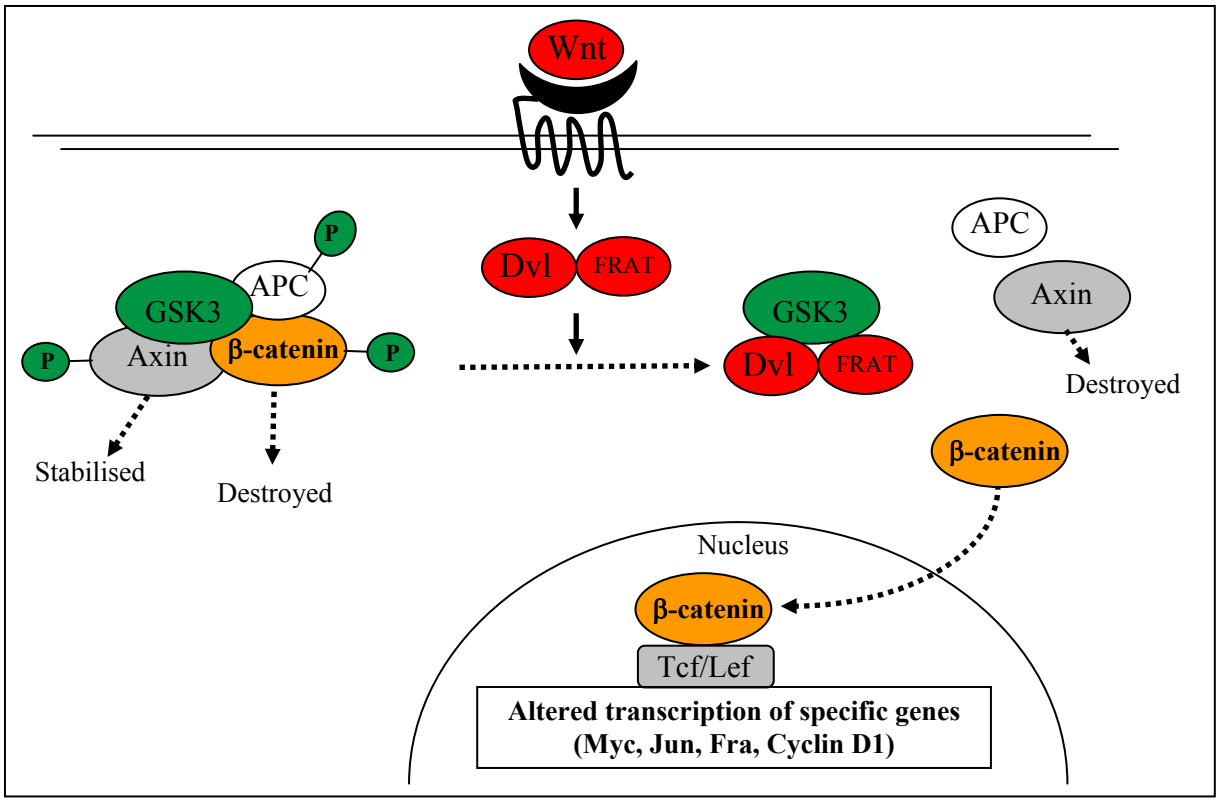


**Figure II - 11: Outline of the mechanism by which Wnt signalling might lead to the inhibition of GSK-3, accumulation of β-catenin and the activation of gene transcription.** This signal transduction pathway has still to be fully elucidated and some of the details depicted are not generally accepted. The Wnt signalling is not only of importance during embryogenesis, but this pathway is also involved in gene regulation in adult organisms, and mutations in some components of the pathway, such as APC and β-catenin, can cause cancer (adapted from Cohen and Goedert, 2004).

Aberrant activation of the canonical Wnt/β-catenin pathway is one of the most frequent signalling abnormalities known in human cancer (Polakis, 2000; Brennan and Brown, 2004). Wnt-1 was the first oncogene identified after infection of mice with the mouse mammary tumor virus (MMTV) and transgenic expression of Wnt-1 in mice induces ER+ mammary tumors (Zhang *et al*., 2005). Aberrant Wnt signalling is also associated with breast tumorigenesis although mutations of β-catenin or other Wnt pathway components have been found only rarely in breast cancer. However, stabilisation and nuclear translocation of β catenin protein has been reported in the majority of human breast tumors (Howe and Brown, 2004). Nuclear β-catenin can function as an oncogene when it binds to TCF family members and increase the expression of genes involved in breast cancer proliferation such as cyclin D1. High β-catenin activity significantly correlated with poor prognosis of breast cancer patients (Lin *et al*., 2000). The cause of β-catenin accumulation has not yet been determined but overexpression of some Wnt glycoproteins (Wnt 2, 3, 4, 5A, 7B, 10B, 13 and 14) has been documented in human breast tissue and may be at the origin of this

accumulation. Altered activity of Wnt signalling modulators such as the protein kinase GSK-3 may also be responsible for β-catenin stabilisation.

### II - 4.4.3) Early findings concerning possible ERα regulation by GSK-3β

ERα acts as a transcription factor and its ligand-dependent regulation is under the influence of phosphorylation. Some protein kinases have already been described to control ERα transcriptional activity (see part II - 4.3) but they failed to explain the whole mechanistic machinery responsible for the effects of estrogen signalling. Therefore, new alternative protein kinase candidates may play an important role. Several evidences indicate that GSK-3β influences ERα function and activity. In rat hippocampus, Cardonna-Gomez *et al.* (2004) observed that estradiol regulates interaction of ERα, GSK-3β and β-catenin. In 2004, Kouzmenko *et al.* suggested a functional interaction between β-catenin and ERα in human colon and breast cancer cells. However, the other components involved in the cross-talk between Wnt, estrogen signalling pathways must still be identified, and GSK-3β is only a putative candidate.

Recently, our group (De Servi *et al.*, 2005) demonstrated that GSK-3β plays a role in TPA-induced or ligand-independent ERα activation and translocation in breast cancer cells (**Fig. II - 12**). In the same cell line, we observed that ERα phosphorylation by GSK-3β stabilises this receptor under resting conditions and modulates ERα transcriptional activity upon ligand binding. This dual action of GSK-3β suggests a key role for this protein kinase in the regulation of the ligand-dependent activation of ERα (Medunjanin *et al*., 2005).

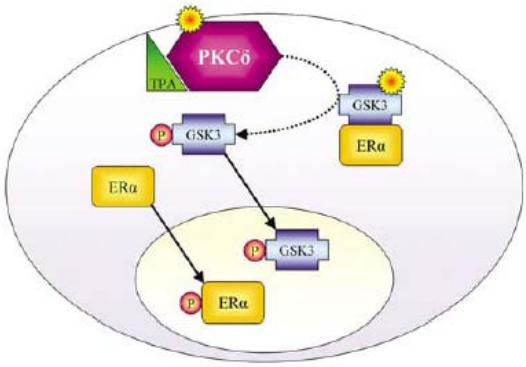


**Figure II - 12: Model for PKCδ involvement in TPA-induced activation of ERα.** In unstimulated cells, PKCδ is localized to the cytoplasm. After TPA treatment, active PKCδ translocates to the membrane fraction and phosphorylates GSK-3. GSK3, which in unstimulated cells is active and forms a complex with ERα, is inactivated by phosphorylation. The phosphorylation of GSK-3 may trigger the nuclear translocation of both ERα and GSK3 proteins. ERα is activated by phosphorylation in the nucleus. (De Servi et al., 2005).

## II - 5) Aims

The majority of breast cancers show estrogen-dependent growth via the activation of the ERα signalling pathway. The observation that ERα negative tumors may arise from ERα positive neoplasms reinforces the significance of estrogens for early breast cancer development. However, open questions are still encountered regarding the mechanism(s) leading to activation of the estrogen-dependent signalling pathway(s) and their particular role in breast tumorigenesis. It is assumed that both nongenomic and genomic actions of E2 via the ERα signalling pathway(s) are involved.

Cytoplasmic proteins, especially protein kinases that are components of various signal transduction pathways, are rapidly phosphorylated upon stimulation of cells with E2. These rapid effects of E2 are considered nongenomic effects. In addition, rapid E2-activated signalling reactions result in translocation of cytoplasmic ERα into the nucleus where phosphorylation in the AF-1 domain of the receptor occurs. This ligand-dependent activation of ERα leads to genomic effects of E2/ERα and triggers the expression of ERE-dependent target genes.

The aim of this study is to unravel the functional relationship of GSK-3 and ERα and to clarify the specific role of GSK-3 in the signalling processes related to E2-dependent ERα activation. A dual role of GSK-3 is postulated with reactions on ERα taking place in both the cytoplasm and the nucleus. In the cytoplasm, GSK-3 is assumed to interact with ERα and to stabilise the receptor. In the nucleus, Ser-118 is considered the major ERα phosphorylation site for GSK-3, phosphorylation leading to full transcriptional activity of the receptor. The studies are performed mainly on MCF-7 cells, a human ERα-positive breast cancer cell line. Furthermore, initial studies regarding GSK-3 expression in human breast cancer specimens of different grade are also performed, the ultimate aim being to investigate the role of the newly depicted GSK-3/ERα pathway for breast cancer progression.

The following aspects are specifically addressed:

- In MCF-7 cells, the rapid effects of E2 treatment on GSK-3 and ERα are first depicted by cellular fractionation and western blot analysis as well as immunofluorescence microscopy. The kinetics of GSK-3 phosphorylation and its sub-cellular localisation are determined upon E2 stimulation. This phosphorylation is assumed to represent non-

genomic effects of the E2/ERα signalling pathway. Additionally, the kinetics of nuclear translocation and Ser-118 phosphorylation of ERα are determined following E2-exposure.

- The major part of this work is dedicated to the clarification of the role of GSK-3 in E2-induced ERα activation in ERα-positive human breast carcinoma cell lines. Different methodological approaches including various chemical GSK-3 inhibitors, cellular transfection of GSK-3β mutants, and RNA interference using siRNA and miR specifically targeting GSK-3α and GSK-3β isoforms are used.
    - The impact of GSK-3 on ERα stability and proteasomal degradation is studied in GSK-3 silenced cells. Rescue of silenced endogenous GSK-3 using xenopus GSK-3 construct shows the role of the docking properties of GSK-3 regarding its interaction with ERα.
    - The role of GSK-3 in E2-induced ERα phosphorylation at Ser-118 is clarified using *in vitro* protein kinase assays and western blot analysis with phospho-specific antibody. In cells and especially in cells treated with GSK-3 inhibitors and in GSK-3 silenced cells, immunoblots performed after cellular fractionation identify the cellular compartment where this reaction takes place.
    - To investigate the impact of GSK-3β on E2-dependent ERα transcriptional activity, ERE-controlled luciferase reporter assays and quantitative analysis of mRNA expression of estrogen-dependent genes (pS2, progesterone receptor) are performed in GSK-3 silenced cells and in cells overexpressing GSK-3β mutants.
- Finally, immunohistochemical staining using a GSK-3β antibody is performed on formalin-fixed human breast tissue sections. The aim is to determine the expression pattern of GSK-3β according to the histopathological grade of tumors surgically removed from patients and to evaluate the relevance of GSK-3β expression for the progression of breast cancer.

# III - Experimental procedures

## III - 1) Materials

### III - 1.1) Equipment

| ▪ Name | ▪ Company |
|---|---|
| Analytical balance 2002 MP1 | Sartorius, Göttingen |
| Balance (max. 5500 g) | Sartorius, Göttingen |
| Blotting chamber for wet blotting (incl. porous pads and plastic support) | Sigma, Deisenhofen |
| Centrifuge GPK | Beckman Instruments, München |
| Centrifuge for Eppendorf tubes Biofuge | Heraeus, Hanau |
| Dichroic Mirror (XF2043) | Omega, Brattleboro, VT, USA |
| Gel Doc XR | Bio Rad, München |
| Heating block Dri Block DB3 | Thermo-Dux, Wertheim |
| Incubator (bacterial culture) | Heraeus, Hanau |
| Luminometer Biolumat LB9505 incl. tubes | Berthold, Bad Wildbad |
| Magnetic stirrer MR 2002 | Heidolph, Kelheim |
| Microscope (light) | Leica Microsystems, Solms |
| Microscope (fluorescence) Axioplan | Zeiss, Oberkochen |
| Microscope camera DFC 480 | Leica Microsystems, Solms |
| Microwave Micromat 135 | AEG, Nürnberg |
| Microtome | Leica Microsystems, Solms |
| Multiskan Ex (Plate Reader) | Thermo Fisher Scientific, Dreieich |
| Neubauer hemacytometer | Schott, Hofheim |
| PCR Primus96 Plus machine | MWG Biotech, Martinsried |
| PTC-200 Peltier Thermal Cycler | MJ Research, Miami, USA |
| pH electrode InLab 410 | Mettler Toledo, Steinbach |
| pH meter 535 Multical | WTW, Weilheim |
| Photometer Ultrospec III | Amersham Pharmacia Biotech, Freiburg |
| Pipetboy acu | Integra, Hamburg |
| Powersupply EPS 300 | Amersham Pharmacia Biotech, Freiburg |
| Refrigerated centrifuge “fresco” | Heraeus, Hanau |
| SDS-PAGE Mini Protean II and III | Bio-Rad, München |
| Shaker for bacterial cultures | Infors HT, Stuttgart |
| Spin-over rotator Bioblock | Novodirect, Kehl |
| Thermomixer compact | Eppendorf, Hamburg |
| Water-purification system Milli-Q | Millipore, Eschwege |
| Vortex mixer | Bender u. Hobein AG, Zürich (Switzerland) |
| X-ray film developer Hyperprocessor | Amersham Pharmacia Biotech, Freiburg |

### III - 1.2) Chemicals and other compounds

| ▪ Name | ▪ Company |
|---|---|
| Acetic acid | Merck, Darmstadt |
| Acrylamide/bis-acrylamide, 30% solution | Sigma, Deisenhofen |
| Adenosine triphosphate (ATP) | Roche, Mannheim |

| | |
|---|---|
| Agarose | Sigma, Deisenhofen |
| Ammonium persulfate | Merck, Darmstadt |
| Ampicillin | Sigma, Deisenhofen |
| Bacto-agar | Merck, Darmstadt |
| Bovine serum albumin, 30% solution | Bio Rad, München |
| Braun $H_2O$ (aqua ad iniectabilia) | Braun, Melsungen |
| Bromophenol blue | Serva, Heidelberg |
| Citric acid | Merck, Darmstadt |
| Complete mini EDTA-free protease inhibitor cocktail tablet | Roche, Mannheim |
| Cycloheximide (CHX) | Sigma, Deisenhofen |
| DC protein assay kit | Bio Rad, München |
| Dimethyl sulphoxide (DMSO) | Merck, Darmstadt |
| Dithiothreitol (DTT) | Sigma, Deisenhofen |
| dNTPs mix | Stratagene, Amsterdam, The Netherlands |
| ECL-plus reagent | Amersham Pharmacia Biotech, Freiburg |
| 17β-estradiol (E2) | Sigma, Deisenhofen |
| Ethanol absolute | Riedel de Haen, Seezle |
| Ethidium bromide | Invitrogen, Karlsruhe |
| Ethylenediaminetetraacetate (EDTA) | Fluka, Buchs (Switzerland) |
| Fugene HD | Roche, Mannheim |
| Glutaraldehyde | Serva, Heidelberg |
| Glycerol | Roth, Karlsruhe |
| Glycine | GERBU Biotech GmbH, Gaiberg |
| HEPES | GERBU Biotech GmbH, Gaiberg |
| HEPES/KOH | Fluka, Buchs (Switzerland) |
| HistoBond adhesion microscope slides | Superior Marienfeld, Lauda-Königshofen |
| Hydrochloric acid (HCl) | Fluka, Buchs (Switzerland) |
| JetPEI™ polyplus transfection | Biomol, Hamburg |
| Luciferase assay reagent (LAR) | Promega, Mannheim |
| Lithium chloride (LiCl) | Fluka, Buchs (Switzerland) |
| Luciferase cell culture lysis 5 x reagent | Promega, Mannheim |
| 2-Mercaptoethanol | Sigma, Deisenhofen |
| Methanol | Merck, Darmstadt |
| Manganese chloride ($MnCl_2$) | Merck, Darmstadt |
| Magnesium chloride ($MgCl_2$) | PeqLab, Erlangen |
| Magnesium sulfate ($MgSO_4$) | Sigma, Deisenhofen |
| MG132 (Proteasome inhibitor) | EMD Bioscience, Darmstadt |
| Milk powder | Fluka, Buchs (Switzerland) |
| Micro RNAs (miR) | Operon, Köln |
| Microtome blades | Thermo Fisher Scientific, Dreieich |
| Oligo(dT) primers | Qiagen, Hilden |
| Oligofectamine | Invitrogen, Karlsruhe |
| Oligonucleotide PCR primers | Operon, Köln |
| 2-nitrophenyl-β-D-galactopyranoside (ONPG) | Biomol, Hamburg |
| Paraformaldehyde (PFA) | Merck, Darmstadt |
| Para-nitrophenyl phosphate | EMD biosciences, Darmstadt |
| PMSF | Sigma, Deisenhofen |
| Poly-D-lysine | BD biosciences, Erembodegem, Belgium |
| Potassium chloride (KCl) | Merck, Darmstadt |

| | |
|---|---|
| Protein A sepharose beads | Sigma, Deisenhofen |
| Protein phosphatase-λ | EMD biosciences, Darmstadt |
| PVDF-membrane Immobilon-P | Millipore, Eschwege |
| *Pwo*-polymerase | PeqLab, Erlangen |
| Recombinant rabbit GSK-3β | New England Biolabs, Frankfurt |
| Recombinant human estrogen receptor α (rhERα) | Invitrogen, Karlsruhe |
| Restriction endonucleases (Bgl II, Bam HI, Nhe I) | Roche, Mannheim |
| RNase inhibitor | Roche, Mannheim |
| SB415286 ($SB_4$) | Tocris, Ballwin, MO, USA |
| SB216763 ($SB_2$) | Tocris, Ballwin, MO, USA |
| Sodium azide ($NaN_3$) | Merck, Darmstadt |
| Sodium chloride (NaCl) | Fluka, Buchs (Switzerland) |
| Sodium citrate | Merck, Darmstadt |
| Sodium carbonate ($Na_2CO_3$) | Merck, Darmstadt |
| Di-sodium-hydrogen-phosphate ($Na_2HPO_4$) | Merck, Darmstadt |
| Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4$) | Merck, Darmstadt |
| Sodium dodecyl sulfate (SDS) | Serva, Heidelberg |
| Sodium fluoride (NaF) | Merck, Darmstadt |
| Sodium hydroxide (NaOH) | Riedel de Haen, Seezle |
| Sodium molybdate | Sigma, Deisenhofen |
| Sodium orthovanadate ($Na_3VO_4$) | Sigma, Deisenhofen |
| Spectinomycin | Sigma, Deisenhofen |
| Superscript II reverse transcriptase | Invitrogen, Karlsruhe |
| SYBR green supermix | Bio Rad, München |
| T4 DNA ligase | Promega, Mannheim |
| *Taq*-polymerase | Invitrogen, Karlsruhe |
| N,N,N',N'-tetramethyl ethylenediamine (TEMED) | Serva, Heidelberg |
| Tris-base | Sigma, Deisenhofen |
| Tris-HCl | Roth, Karlsruhe |
| Triton X-100 | Serva, Heidelberg |
| Trypan blue | Biochrom, Berlin |
| Trypton | Difco, Detroit, USA |
| Tween-20 | GERBU, Gaiberg |
| Yeast extract | Difco, Detroit, USA |
| X-ray film | Konica, Tokyo (Japan) |
| Xylene | Fluka, Buchs (Switzerland) |

Disposable materials such as laboratory plastic and reaction tubes were purchased from Becton Dickinson (Franklin Lakes, New Jersey, USA), Bio-Rad (München) Eppendorf (Hamburg), Greiner (Frickenhausen) and Sarstedt (Nümbrecht).

### III - 1.3) Solutions and media for bacterial culture

| ▪ **Specification** | ▪ **Composition** | |
|---|---|---|
| ▪ **LB medium (pH 7,4)** | 1 % (w/v) | NaCl |
| | 1 % (w/v) | Yeast extract |
| | 0,5 % (w/v) | Trypton |
| | | in distilled $H_2O$ |

| | | |
|---|---|---|
| ▪ **LB agar (pH 7,4)** | 1,5 % (w/v)<br>100 µg/ml | bacto-agar in LB medium<br>Ampicillin |
| ▪ **Ampicillin stock solution** | 10 mg/ml | Ampicillin in distilled $H_2O$ |
| ▪ **Spectinomycin** | 10 mg/ml | Spectinomycin in distilled $H_2O$ |

### III - 1.4) Molecular biology kits

| Kit | Description | Company |
|---|---|---|
| One Shot TOP10 chemically competent cells | Transformation of bacteria with plasmid DNA to amplify the vector construct | Invitrogen, Karlsruhe |
| QIAprep miniprep/maxiprep | Plasmid DNA extraction from bacteria | Qiagen, Hilden |
| QIAquick gel extraction | Extraction of nucleotide fragments from agarose gels | Qiagen, Hilden |
| QIAquick PCR purification and nucleotide removal | To cleanup DNA fragments from enzymatic reactions | Qiagen, Hilden |
| QuickChange site-directed mutagenesis kit | To make point mutations, switch amino acids, delete or insert single or multiple amino acids in a vector construct sequence | Stratagene, Amsterdam, The Netherlands |
| RNeasy | Extraction of total RNA from cells or tissues | Qiagen, Hilden |
| BLOCK-iT Pol II miR RNAi expression vector kits | Gateway-adapted expression vector for the RNAi analysis of a target gene in mammalian cells | Invitrogen, Karlsruhe |

**Table III - 1: Commercially available kits.**

### III - 1.5) Plasmid constructs

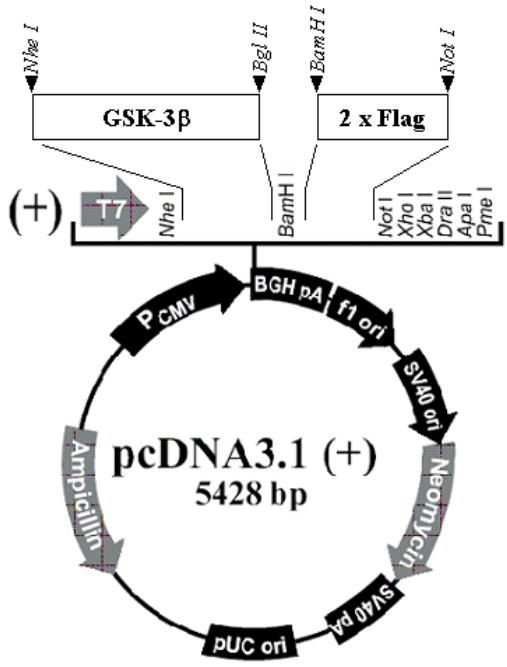


**Figure III - 1: pcDNA3.1 construct map.** GSK-3β was cloned in a pcDNA3.1(+) vector from Invitrogen and tagged with 2 x Flag. The restriction enzymes used are written in italics at the beginning and the end of every inserts (adapted from pcDNA3.1 Vectors Datasheet, Invitrogen).

| Plasmid name | Vector | Insert | Plasmid size [bp] | Source |
|---|---|---|---|---|
| pcDNA GSK-3β WT | pcDNA3.1/2xFlag | Human GSK-3β | 6793 | Senad Medunjanin |
| pcDNA GSK-3β CA | pcDNA3.1/2xFlag | Human GSK-3β S9A | 6793 | Senad Medunjanin |
| pcDNA GSK-3β R96A | pcDNA3.1/2xFlag | Human GSK-3β R96A | 6793 | - |
| pcDNA GSK-3β R96K | pcDNA3.1/2xFlag | Human GSK-3β R96K | 6793 | - |
| pCMV β-gal | pCMV-Script | β-galactosidase | 7164 | Margit Klier |
| pCS2+ GSK-3β WT | pCS2+ | Xenopus GSK-3β | 5400 | Gary Davidson |
| pCS2+ GSK-3β DN | pCS2+ | Xenopus GSK-3β Dominant Negative | 5400 | Gary Davidson |

**Table III - 2: Expression plasmid DNA constructs.**

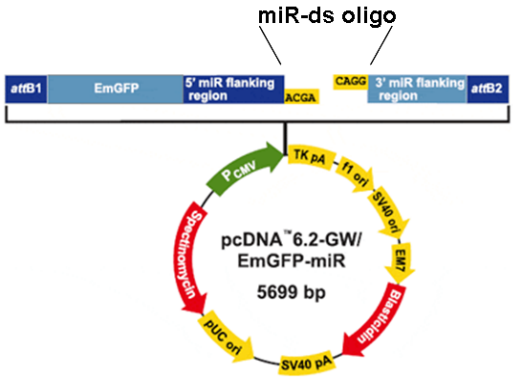


**Figure III - 2: pcDNA6.2-GW/EmGFP-miR construct map.** Double stranded microRNA oligonucleotides (miR-ds oligo) targeting GSK-3β were inserted into pcDNA6.2-GW/EmGFP vector (adapted from BLOCK-iT Pol II miR RNAi expression vector kits datasheet, Invitrogen).

| Plasmid name | Vector | miR-ds oligo sequence (5' → 3') | Plasmid size [bp] |
|---|---|---|---|
| CT miR | pcDNA6.2-GW/EGFP-miR | *Top strand:* TGCTGTTAACTAAACCATTAACCACCGTTTTGGCCA<br>*Bottom strand:* CCTGTTAACTAAACCTAACCACCGTCAGTCAGTGGC | 5735 |
| GSK-3β miR 741 | pcDNA6.2-GW/EGFP-miR | *Top strand:* TGCTGTAGATGATTTAGGACTTGGGAGTTTTGGCCACT GACTGACTCCCAAGTTAAATCTCTA<br>*Bottom strand:* CCTGTAGATGATTTAACTTGGGAGTCAGTCAGTGGC CAAAACTCCCAAGTCCTAAATCATCTAC | 5762 |
| GSK-3β miR 599 | pcDNA6.2-GW/EGFP-miR | *Top strand:* TGCTGTTATATTTTCCTCGGGGATTTGTTTTGGCCACTG ACTGACAAATCCCCGGAAAATATAA<br>*Bottom strand:* CCTGTTATATTTTCCGGGGATTTGTCAGTCAGT GGC CAAAACAAATCCCCGAGGAAAATATAAC | 5762 |
| GSK-3β miR 1839 | pcDNA6.2-GW/EGFP-miR | *Top strand:* TGCTGTAAATTCTGTGTAGTTTGGGTGTTTTGGCCACTG ACTGACACCCAAACCACAGAATTTA<br>*Bottom strand:* CCTGTAAATTCTGTGGTTTGGGTGTCAGTCAGTGGC CAAAACACCCAAACTACACAGAATTTAC | 5762 |

**Table III - 3: microRNA expression vectors.** The negative control (CT miR) targeting a mouse brain receptor (GPR54) was kindly provided by Milen Kirilov and was used as reference. GSK-3β miR 741, GSK-3β miR 599 and GSK-3β miR 1839 were designed to target the human GSK-3β.

### III - 1.6) Small interfering RNA (siRNA) sequences

| Targets | siRNA references and/or sequences | Company |
|---|---|---|
| GL3 | Luciferase GL3 duplex siRNA | Dharmacon, Lafayette, CO, USA |
| GSK-3α/β | Signal Silencing GSK-3α/β siRNA | New England Biolabs, Frankfurt |
| GSK-3α | Signal Silence GSK-3α siRNA | New England Biolabs, Frankfurt |
| GSK-3β | validated siRNA (ID # 42839) | Ambion, Austin, TX, USA |
| GSK-3β | siRNA duplex: 5'-AUCUUUGGAGCCACUGAUU-3' (Phiel *et al*. 2003) | Dharmacon, Lafayette, CO, USA |

**Table III - 3: List of siRNA constructs.**

### III - 1.7) PCR primers

| Name | Specificity | Organism | Length [N] | Sequence (5' → 3') | Tm [°C] |
|---|---|---|---|---|---|
| SYBR β-actin Fwd | β–actin | H. Sapiens | 18 | CCAACCGCGAGAAGATGA | 59,9 |
| SYBR β-actin Rev | β–actin | H. Sapiens | 20 | CCAGAGGCGTACAGGGATAG | 64,5 |
| SYBR ERα Fwd | ERα | H. Sapiens | 20 | TTACTGACCAACCTGGCAGA | 60,4 |
| SYBR ERα Rev | ERα | H. Sapiens | 20 | ATCATGGAGGGTCAAATCCA | 58,4 |
| SYBR GSK-3β Fwd | GSK-3β | H. Sapiens | 21 | GACATTTCACCTCAGGAGTGC | 62,6 |
| SYBR GSK-3β Rev | GSK-3β | H. Sapiens | 20 | GTTAGTCGGGCAGTTGGTGT | 62,5 |
| GSK-3β WT Fwd | GSK-3β | H. Sapiens | 34 | TATAGCTAGCATGTCAGGGCGGC CCAGAACCACC | 64,2 |
| GSK-3β WT Rev | GSK-3β | H. Sapiens | 34 | TATAAGATCTGGAGGAGTTGGAA GCTGATGCAGA | 58,1 |
| GSK-3β R96A Fwd | GSK-3β R96A | H. Sapiens | 25 | GCGATTTAAGAACGCAGAGCTCC AG | 66,2 |
| GSK-3β R96A Rev | GSK-3β R96A | H. Sapiens | 25 | CTGGAGCTCTGCGTTCTTAAATC GC | 66,2 |
| GSK-3β R96K Fwd | GSK-3β R96K | H. Sapiens | 28 | GCGATTTAAGAACAAAGAGCTCC AGATC | 64,6 |
| GSK-3β R96K Rev | GSK-3β R96K | H. Sapiens | 28 | GATCTGGAGCTCTTTGTTCTTAAA TCGC | 64,6 |
| SYBR pS2 Fwd | pS2 | H. Sapiens | 20 | ATACCATCGACGTCCCTCCA | 62,5 |
| SYBR pS2 Rev | pS2 | H. Sapiens | 20 | AAGCGTGTCTGAGGTGTCCG | 64,5 |
| SYBR PR Fwd | PR Receptor | H. Sapiens | 18 | GGCATGGTCCTTGGAGGT | 62,2 |
| SYBR PR Rev | PR Receptor | H. Sapiens | 18 | CAATGGCTGTGGGAGAGC | 62,2 |

**Table III - 4: Oligonucleotides used for PCR and quantitative real-time PCR.**

### III - 1.8) Buffers and solutions for agarose gel electrophoresis

| ▪ **Specification** | **Composition** | |
|---|---|---|
| ▪ **TAE buffer (50 x, 1l)** | 2 M | Tris |
| | 57 ml | Glacial Acetic Acid |
| | 100 ml | 0,5 M EDTA (pH 8,0) |
| | | in distilled $H_2O$ |
| ▪ **Ethidium Bromide** | 10 mg/ml (Invitrogen) | |
| ▪ **TE buffer (pH 8,0)** | 10 mM | Tris-HCl |
| | 1 M | EDTA |
| ▪ **Agarose gel (100 ml)** | 1 g | Agarose (Sigma) in 1 x TAE buffer (DNA) |
| ▪ **Sample Buffer (6 x)** | 100 mM | EDTA (pH 8,0) |
| | 30 % (v/v) | Glycerol |
| | 0,025 % | Bromophenol Blue |
| | 0,025 % | Xylene Cyanol |
| ▪ **DNA marker** | DNA molecular weight marker VII and XVI (Roche) | |

### III - 1.9) Cell culture equipments and chemicals

| ▪ **Name** | ▪ **Company** |
|---|---|
| Dextran 60 | Serva, Heidelberg |
| Dulbecco's MEM, 3,7 g/l NaHCO3, 4,5 g/l D-glucose, phenol-red free, glutamine-free, pyruvate-free (DMEM) | Biochrom, Berlin |
| EDTA 1 % (w/v) in PBS | Biochrom, Berlin |
| Falcon tissue culture plates (96-wells, 6 wells and 10 cm diameter) | Becton Dickinson, Heidelberg |
| Falcon tubes (15 ml, 50 ml) polystyrene | Becton Dickinson, Heidelberg |
| Foetal calf serum (FCS) | Biochrom, Berlin |
| Humidified $CO_2$ incubator | Forma Scientific, Labotech Göttingen |
| L-glutamine, 200 mM | Biochrom, Berlin |
| Norrit A (charcoal) | Serva, Heidelberg |
| Penicillin/streptomycin (10.000 U/10.000 µg/ml) | Biochrom Berlin |
| RPMI 1640, phenol-red free, glutamine-free, pyruvate-free (RPMI) | PAA, Linz, Austria |
| Sodium pyruvate (1 M) | Biochrom, Berlin) |
| Sterile bench | Heraeus, Hanau |
| Trypsin/EDTA, 0,05 %/0,02 % (w/v) in PBS | Biochrom, Berlin |

### III - 1.10) Cell lines

| Cell line | Medium | Source - Description |
|---|---|---|
| MCF-7 | DMEM | Human (ER+) epithelial breast adenocarcinoma cells derived from metastatic pleural effusion (DSMZ, Braunschweig) |
| MELN | DMEM | MCF-7 cells (ER+) stably transfected with ERE-controlled luciferase reporter plasmid (Le Bail *et al*., 1998) |
| T47D | RPMI | Human (ER+) epithelial breast ductal carcinoma cells derived from metastatic pleural effusion (ATCC, Wesel) |
| BT-474 | RPMI | Human (ER+) epithelial breast ductal carcinoma cells isolated from solid invasive tumour (ATCC, Wesel) |

**Table III - 5: Culture cell lines used.**

### III - 1.11) Solutions and media for cell culture

| ▪ Specification | ▪ Composition |
|---|---|
| ▪ **M2** | phenol-red free DMEM or RPMI<br>100 U/ml resp. 100 µg/ml Penicillin/Streptomycin<br>10 % FCS<br>1 mM sodium pyruvate<br>2 mM L-glutamine |
| ▪ **M3** | phenol-red free DMEM or RPMI<br>100 U/ml resp. 100 µg/ml Penicillin/Streptomycin<br>10 % dextran-coated charcoal-treated FCS (DCC-FCS)<br>1 mM sodium pyruvate<br>2 mM L-glutamine |
| ▪ **M4** | phenol-red free DMEM<br>100 U/ml resp. 100 µg/ml Penicillin/Streptomycin<br>1 mM sodium pyruvate<br>2 mM L-glutamine |
| ▪ **DCC solution** | 0,01 M Tris-HCl (pH 8,0)<br>0,25 % (w/v) Charcoal Norrit A<br>0,0025 % (w/v) Dextran 60<br>in distilled $H_2O$ |
| ▪ **EDTA** | 1 % EDTA in PBS |
| ▪ **Trypsin/EDTA** | 0,05 % Trypsin<br>0,02 % EDTA in PBS |
| ▪ **PBS (10x)** | 0,86 M NaCl<br>0,58 M $Na_2HPO_4$<br>0,17 M $NaH_2PO_4 \times 2H_2O$<br>in distilled $H_2O$<br>(pH 7,4 was adjusted in 1 x PBS working solution) |

| | | |
|---|---|---|
| ▪ **Lysis Buffer** | 50 mM | HEPES (pH 7,6) |
| | 150 mM | NaCl |
| | 1,5 mM | $MgCl_2$ |
| | 10 mM | $Na_4P_2O_7$ x 10 $H_2O$ |
| | 2 mM | EDTA |
| | 10 % (v/v) | Glycerol |
| | 1,5 % (v/v) | Triton X-100 |
| | 100 mM | Na-Fluoride |
| | 2,7 mM | Na-Orthovanadate ($Na_3VO_4$) |
| | 1 (/10 ml) | Protease Inhibitor Cocktail Tablet |
| | | in distilled $H_2O$ |
| ▪ **Buffer I** | 10 mM | HEPES/KOH (pH 7,9) |
| | 1,5 mM | $MgCl_2$ |
| | 10 mM | KCl |
| | 0,5 mM | DTT |
| | 0,2 mM | PMSF |
| | 1 mM | $Na_3VO_4$ |
| | 1 (/10 ml) | Protease Inhibitor Cocktail Tablet |
| | | in distilled $H_2O$ |
| ▪ **Buffer II** | 20 mM | HEPES/KOH (pH 7,9) |
| | 1,5 mM | $MgCl_2$ |
| | 420 mM | NaCl |
| | 0,2 mM | EDTA |
| | 0,5 mM | DTT |
| | 0,2 mM | PMSF |
| | 1 mM | $Na_3VO_4$ |
| | 0,5 % (v/v) | Glycerol |
| | | in distilled $H_2O$ |

### III - 1.12) Buffers and solutions for western blot

| ▪ **Specification** | ▪ **Composition** | |
|---|---|---|
| ▪ **Sample buffer (5 x)** | 50 mM | Tris-HCl (pH 6,7) |
| | 200 mM | DTT |
| | 2 % | SDS |
| | 2,5 % | Glycerol |
| | 0,1 % | Bromophenol blue |
| | 1,75 M | β-Mercaptoethanol |
| ▪ **TBS (10 x)** | 0,1 M | Tris-HCl (pH 7,6) |
| | 1,5 M | NaCl |
| ▪ **TBS/T** | 1 x | TBS |
| | 0,1 % | Tween |
| ▪ **Tris-buffer (stacking gel)** | 1,5 M | Tris-HCl (pH 8,8) |
| | 0,4 % | SDS |

| | | |
|---|---|---|
| ▪ **Tris-buffer** | 1 M | Tris-HCl (pH 6,8) |
| **(separation gel)** | 0,4 % | SDS |
| ▪ **Acrylamide solution** | 30:0,8 | acrylamide/bisacrylamide |
| ▪ **APS** | 10 % (w/v) | Ammonium Persulfat in distilled $H_2O$ |
| ▪ **SDS-PAGE gel** | 4,02 ml | $H_2O$ |
| (10 %, 2 mini gels) | 3,3 ml | 30 % acrylamide solution |
| | 2,5 ml | 1,5 M Tris (pH 8,8) |
| | 100 µl | 10 % SDS |
| | 50 µl | 10 % Ammonium Persulfat |
| | 30 µl | Temed |
| ▪ **Stacking gel** | 3 ml | $H_2O$ |
| (5 %, 2 mini gels) | 0,66 ml | 30 % acrylamide solution |
| | 1,26 ml | 1 M Tris (pH 6,8) |
| | 50 µl | 10 % SDS |
| | 38 µl | 10 % Ammonium Persulfat |
| | 30 µl | Temed |
| ▪ **Running buffer (1 x)** | 25 mM | Tris-HCl (pH 8,3) |
| | 250 mM | Glycine |
| | 0,1 % | SDS |
| ▪ **Protein Standard** | Protein All Blue precision plus standard (Bio-Rad) | |
| ▪ **Transfer buffer** | 50 mM | Tris-base |
| | 380 mM | Glycine |
| | 20 % | Methanol |
| ▪ **Blocking buffer** | 5 % | Non-fat dry milk in TBS/T |
| ▪ **Stripping buffer** | 62 mM | Tris-HCl (pH 6,7) |
| | 7,18 ml | β-Mercaptoethanol |
| | 200 ml | 10 % SDS |
| | | adjust to 1 l with distilled $H_2O$ |

## III - 1.13) Antibodies

### III - 1.13.1) Primary antibody

| Specification | Clone | Species | Sub-class | Dilution WB | IF | IHC | Company supplier |
|---|---|---|---|---|---|---|---|
| α β-actin | AC-15 | mouse monoclonal | IgG | 1:200000 | - | - | Abcam Cambridge, UK |
| α β-catenin | | rabbit polyclonal | IgG | 1:500 | - | - | Cell Signalling NEB, Frankfurt |
| α phospho-β-catenin (Ser-33/37/Thr-41) | | rabbit polyclonal | IgG | 1:500 | - | - | Cell Signalling NEB, Frankfurt |
| α cyclin D1 | H-295 | rabbit polyclonal | IgG | 1:200 | - | - | Santa Cruz Biot. Heidelberg |
| α phospho-cyclin D1 (Thr-286) | | rabbit polyclonal | IgG | 1:750 | - | - | Cell Signalling NEB, Frankfurt |
| α ERα | HC-20 | rabbit polyclonal | IgG | 1:1000 | 1:100 | - | Santa Cruz Biot. Heidelberg |
| α ERα | 6F11 | mouse monoclonal | IgG | 1:1000 | - | - | Novocastra Newcastle, UK |
| α phosphor-ERα (Ser-118) | 16J4 | mouse monoclonal | IgG | 1:5000 | 1:100 | - | Cell Signalling NEB, Frankfurt |
| α Flag-tag | | mouse monoclonal | IgG | 1:20000 | - | - | Sigma München |
| α GSK-3α/β | | mouse monoclonal | IgG | 1:50000 | 1:100 | - | Biosource Solingen |
| α GSK-3β | | rabbit monoclonal | IgG | 1:800 | - | 1:100 | Cell Signalling NEB, Frankfurt |
| α phospho-GSK-3α/β (Ser-21/9) | | rabbit polyclonal | IgG | 1:800 | 1:100 | - | Cell Signalling NEB, Frankfurt |
| α RPA / p34 (replication protein ) | 9H8 or 34A | mouse monoclonal | IgG | 1:200 | - | - | Lab Vision CA, USA |
| α β-tubulin | 2G10 | mouse monoclonal | IgG | 1:10000 | - | - | Upstate NY, USA |
| α ubiquitin | | rabbit polyclonal | IgG | 1:1000 | - | - | DAKO Glostrup Denmark |

**Table III - 6: List of primary antibodies.**

### III - 1.13.2) Secondary/labelled antibody

| Enzyme/dye | Specificity | Species | Sub-class | WorkingDilution | Company supplier |
|---|---|---|---|---|---|
| HRP | α mouse | goat | IgG | 1:5000 (WB) | Dianova Hamburg |
| HRP | α rabbit | goat | IgG | 1:20000 (WB) | Dianova Hamburg |
| Alexa 488 | α mouse | goat | IgG | 1:400 (IF) | Invitrogen Karlsruhe |
| Cy3 | α rabbit | goat | IgG | 1:300 (IF) | Invitrogen Karlsruhe |

**Table III - 7: List of secondary antibodies.**

### III - 1.14) Buffers and solutions for β-galactosidase activity measurement

| ▪ Specification | ▪ Composition | |
|---|---|---|
| ▪ **LacZ reaction buffer** | 100 mM<br>10 mM<br>1 mM<br>Add freshly β-Mercaptoethanol (35µl/10ml LacZ reaction buffer) | Na-Phosphate pH 7,4<br>KCl<br>$MgSO_4$ |
| ▪ **ONPG solution** | 100 mM<br>4 mg/ml | Na-Phosphate pH 7,0<br>ONPG |
| ▪ **Stop solution** | 1 M | $Na_2CO_3$ |

### III - 1.15) Buffers and solutions for immunofluorescence analysis

| ▪ Specification | ▪ Composition | |
|---|---|---|
| ▪ **Fixation buffer** | 4 % (w/v) | PFA in PBS<br>Dissolved at 65°C and sterile filtrated |
| ▪ **Permeabilisation buffer** | 0,2 % | Triton X-100 in PBS |
| ▪ **Blocking buffer** | 1 % | BSA in PBS |
| ▪ **Mounting media** | Elvanol (Merck) | |

### III - 1.16) Buffers and solutions for immunohistochemistry

| ▪ Specification | ▪ Composition | |
|---|---|---|
| ▪ **Citrate buffer** | 0,01 M | Sodium citrate/citric acid (pH 6,0) |
| ▪ **Blocking buffer** | 5 % | BSA in PBS |
| ▪ **$2^{ary}$ Antibody staining** | LSAB2-kit (DAKO, Glostrup, Denmark) | |
| ▪ **Counter staining** | Haematoxylin (Mayer's Hemalum, AppliChem, Darmstadt) | |
| ▪ **Eosin** | 0,5 %<br>1 drop | Eosin Y (Merck, Darmstadt)<br>Acetic Acid<br>adjust to 100 ml with distilled $H_2O$ |
| ▪ **Mounting media** | Kaiser's glycerol/gelatin (Merck, Darmstadt) | |

## III - 2) Methods

### III - 2.1) Molecular biology

#### III - 2.1.1) RNA isolation and cDNA synthesis

After treatment, cell pellets were collected by trypsinisation and total RNA was isolated with the RNeasy kit. First, the cell pellets were thoroughly resuspended in lysis buffer and homogenised by passing the cells through a RNase-free 20-gauge needle using a syringe. 70 % ethanol was added and the lysate was loaded onto RNeasy mini column. After centrifugation and washing, the RNA retained in the column was eluted in RNase-free water. The RNA concentration was determined and the samples were stored at - 80°C.

1 µg RNA was reverse transcribed using 10 µg/ml (final concentration) oligo(dT) primers. After incubation of RNA/oligo(dT) mix (74°C, 4 min), a mastermix containing at final concentration 0,02 M DTT, 2 U/µl protector RNase inhibitor, 0,5 mM dNTPs, 1 x enzyme buffer and 10 U/µl SuperScript II reverse transcriptase was added. After incubation program (37°C, 2 min; 42°C, 5 min; 50°C, 1 h and 65°C, 15 min), cDNA was purified with the QIAquick PCR purification kit and stored at – 20°C.

#### III - 2.1.2) Determination of nucleic acid concentration

A dilution (1/20 to 1/100) in Braun $H_2O$ was performed on all the nucleic acid samples. Diluted samples were placed in a silica cuvette. Absorbance was then read against a Braun $H_2O$ blank with a spectrophotometer at 260 nm and 280 nm.

Assuming that an absorbance of one unit at 260 nm is equivalent to 40 ng/µl of single stranded RNA, the following equation was applied to calculate the RNA concentration in the samples:

$$[\text{RNA in } \mu g/ml] = \lambda_{260nm} \text{ x dilution factor x } 40$$

Assuming that an absorbance of one unit at 260 nm is equivalent to 50 ng/µl double stranded DNA, the following equation was applied to calculate the DNA concentration in the samples:

$$[\text{DNA in } \mu g/ml] = \lambda_{260nm} \text{ x dilution factor x } 50$$

A ratio of $\lambda_{260\ nm} / \lambda_{280\ nm}$ between 1.5 and 2.0 indicates a satisfactory purity of the DNA or the RNA extracted.

### III - 2.1.3) Polymerase chain reaction (PCR)

Specific PCR thin wall tubes with flat caps (Scientific-labs) were used. 2 µl of cDNA were mixed with a mastermix composed of 1 x polymerase buffer, 1,25 mM $MgCl_2$, 0,15 mM dNTPs, 0,4 µM forward (Fwd) primer, 0,4 µM reverse (Rev) primer at final concentration and volume was adjusted with Braun $H_2O$. Finally, 0,02 U/µl polymerase was added and the tubes were placed into PCR thermal cycler. The cycler program depends on the primers, the polymerase and the final product expected. For the selection of gene-specific primers an online application "Primer3 application" (Whitehead Institute for Biomedical Research, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used. This program gives advice on the required annealing temperature. Primers were synthesised by Operon. Indeed, each cycle of the program is constituted by a DNA denaturation step at 94°C, an annealing step (generally between 55°C and 65°C depending on the set of primers) and an extension step at a temperature depending on the polymerase used (72°C for the *Taq*-polymerase). Cycle number was generally set to 25-35 in the case of semi-quantitative RT-PCR analysis to check the cDNA synthesised and to compare the expression levels of RNA after different treatments of the cells. For cloning, sequences were amplified up to 40 cycles using *P*wo polymerase which contained a proof-reading activity. The amplified DNA was finally checked with an agarose gel electrophoresis after determination of the DNA concentration.

For quantitative PCR, optimal sets of primers were designed for each studied gene using the "ProbeFinder" (Roche Applied Bioscience) website interface (http://qpcr2.probefinder.com) and synthesised by Operon. Quantitative PCR using 5 µl cDNA (from 1:10 dilution), 1 µl SYBR Fwd primer (from 10 µM stock), 1 µl SYBR Rev primer (from 10 µM stock), 3 µl $MgCl_2$ (from 25 mM stock) and 10 µl of iQ SYBR Green supermix were performed following the manufacturers' instructions on PTC-200 Peltier Thermal Cycler and the MJ OpticonMonitor analysis software, version 3.1 (Bio Rad).

### III - 2.1.4) Agarose gel electrophoresis

PCR samples were run on a 1 % (w/v) agarose gel. In a sterilised beaker, 1 g of agarose was added to 100 ml of 1 x TAE (for DNA), heated in a microwave oven, and 4µl of ethidium bromide was added to the agarose solution after it cooled down (around 60°C). Then, the solution was poured into a gel tray with slot former (comb). When the gel had polymerised, it was placed in an electrophoresis tank filled with running buffer. The DNA ladder and the samples supplemented with 1 x loading buffer (final concentration) were

loaded onto the gel and separated by electrophoresis (100 V, 1 h). The gel was finally observed using UV light and photographed (Gel Doc XR device and software, Bio Rad).

### III - 2.1.5) Cloning using restriction endonucleases

For the directed cloning of DNA sequences, the insert sequence extremities were flanked during PCR by two different restriction sites. The two class II restriction endonucleases specific for these sites were used also for linearization of the pcDNA3.1 vector to create "sticky ends". The restriction of the vector and the insert sequence was carried out overnight (O/N) at 37°C and stopped by heating for 10min at 65°C. Then, the DNA was purified from short oligonucleotides using the nucleotide removal kit for the insert sequence. Gel electrophoresis was performed to get rid of undigested vector. The linearized vector was excised from the gel and purified with the QIAquick Gel Extraction Kit. Subsequently, insert and vector were added to a ligation reaction mix in an appropriate molar ratio (usually 3:1) and ligation was carried out O/N at 16°C using T4 DNA ligase. After stopping the reaction by heating at 65°C for 10 min the ligation product was ready for transformation of competent bacteria.

### III - 2.1.6) Cloning of DNA expression vectors with miR for RNAi

Cloning of micro RNAs (miR) into DNA expression vectors for RNAi (RNA interference) was performed using the BLOCK-iT Pol II miR RNAi expression vector kit. First, candidates micro RNA (miR) targeting GSK-3β were selected using the Wadsworth Bioinformatics Centre website interface (http://sfold.wadsworth.org). Then, complementary DNA oligonucleotides containing the sequence coding for the required miR were designed following the manual's instructions from the kit mentioned above and synthesised by Operon. After annealing of the DNA oligonucleotides, the resulting dsDNA was cloned into the pcDNA6.2-GW/EmGFP expression vector using T4 DNA ligase (provided in the kit) and competent bacteria were transformed.

### III - 2.1.7) Single or multiple site mutations

To analyse the effects of amino acids changes on the functions of proteins and especially kinases, single or multiple site mutations were performed using the QuickChange site-directed mutagenesis kit. A proof-reading *Pfu Turbo* DNA polymerase was used to replicate both plasmid strands with high fidelity without displacing the mutant oligonucleotide primers. Indeed, plasmid constructs containing the wild-type inserts were amplified by PCR

with two synthetic oligonucleotide primers containing the desired mutation. Each primer was extended by the polymerase and the mutant plasmid products were treated with *DpnI*. This endonuclease will digest the parental non-mutated DNA. Then, the vector containing the desired mutation will be transformed into supercompetent cells, amplified and finally analysed by sequencing.

#### III - 2.1.8) Transformation of competent bacteria with plasmid DNA

1-10 µl of ligation reaction or 1 µl (1-10 ng) of purified plasmid DNA were added on competent TOP10 bacteria previously thawed on ice. After gentle mix, the bacteria suspension was incubated for 30 min on ice. Then, the bacteria were submitted to a heat shock at 42°C for 42 sec and placed on ice for 2 min. 250µl of pre-warmed SOC medium was added and the bacteria were incubated (37°C for 60 min). 100µl of transformed bacteria were plated on LB-antibiotic agar plates and incubated O/N at 37°C.

#### III - 2.1.9) DNA mini- and maxi-prep

Isolated transformed bacteria colonies were amplified in 5 ml or 200 ml LB-antibiotic culture media, respectively for mini-prep and maxi-prep. After O/N incubation (37°C, 225 rpm), the bacteria were centrifuged (10 min at 6000 x g, 4°C). A glycerol stock of transformed bacteria can be stored at - 80°C for later amplification (850 µl of bacterial culture with 150 µl of 100 % glycerol). From the bacterial pellet, mini-prep and maxi-prep were performed according to the manufacturer's instruction (Qiagen kits). The extracted plasmid DNA was dissolved in TE buffer and stored at - 20°C. For each purified construct, the insert DNA sequence was determined by A. Hunziker (Oligonucleotides synthesis and DNA sequencing group, DKFZ) and checked by comparison of nucleotide sequences alignment using the "Heidelberg UNIX Sequence Analysis Resources" (HUSAR, http://genome.dkfz-heidelberg.de) interface and database research (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov)

### III - 2.2) Cell culture

#### III - 2.2.1) Cell culture media and treatments

MCF-7 and MELN cells were maintained routinely (5 % $CO_2$, 37°C) in M2 medium. To freeze the cells in liquid nitrogen (Working stock in Nunc cryovial, 1 x $10^6$ cells/ml/vial), 10 % DMSO in M2 medium was added to trypsinised cells and the following freezing steps

were performed: 2 h on ice, 6 h at - 20°C, 24 h at - 80°C (at this temperature, the cells can also be stored for 6 months) and finally in liquid nitrogen tank for long-term storage.

Prior to experimental use, cells maintained in M2 medium were grown for 72 h in M3 medium. M3 medium was supplemented with 10 % DCC-FCS. The serum was prepared as described by Migliaccio *et al*. (1993). The DCC suspension was centrifuged for 10 min at 15000 x g. 500 ml FCS was mixed with the pellet obtained from 1 l of DCC suspension and incubated at 56°C for 30 min in order to remove the steroids. Then, the DCC-FCS was centrifuged for 10 min at 15000 x g. The supernatant was collected and the procedure was repeated once more, but the incubation was this time performed at 37°C. Finally, the collected serum was filtered using a 0,22 µm pore size filter, aliquoted and frozen at -20°C.

If not indicated, the following standard protocol was used. 3 x $10^5$ cells were plated in 6-well plates and grown for 24 h in M3 medium. Prior to hormonal treatment with 10 nM or 100 nM E2, M4 medium was added to the cells for 24 h and cells were kept in this medium until the end of the experiment. 1 h or 6 h prior to long term (6 h) and short term (20 min) E2 treatment, respectively, 5 µM (final concentration) of proteasome inhibitor MG132 was added. 50 µg/ml (final concentration) of translation inhibitor cycloheximide was added 1 h prior to hormonal treatment. Different GSK-3β inhibitors were tested by direct addition to the cell culture media. The cells were treated with 30 mM LiCl, 30 µM of SB415286 and 5 µM of SB216763 for the time point indicated in each experiment.

### III - 2.2.2) Cell proliferation study

After different treatment times, the cells were collected by trypsinisation and resuspended in medium. Cell number was determined after staining an aliquot of the cell suspension by addition of 0,05 % (w/v) Trypan Blue solution (1:2 ratio). Under the microscope in a bright-line hemacytometer, living cells respectively white cells were counted to obtain the number of cells per ml. Counting the cells at various time points will allow to draw a proliferation curve of the cells according to the treatment.

### III - 2.2.3) Lysis of cells

After treatment and washing with PBS, the cells were scraped off the culture dish in cold PBS. Then, the cells were transferred into sterile Eppendorf tubes and centrifuged for 5 min at 2000 rpm, 4°C. The supernatant was discarded. For total cell lysate preparation, cell pellet was suspended in ice-cold lysis buffer, incubated on ice for 30 min and centrifuged for 10 min at 13000 rpm, 4°C. The supernatant represents the lysate and it was stored at - 80°C.

### III - 2.2.4) Preparation of cytoplasmic and nuclear cell extracts

After treatment, a cell pellet was obtained (cf. lysis of cells). Cells were incubated on ice for 15 min in hypo-osmotic Buffer I, homogenized using a glass potter and centrifuged (2000 × g, 5 min). The resulting pellets were used later to generate nuclear extracts. The supernatants were centrifuged (10000 × g, 30 min) and the resulting supernatants were used as the cytoplasmic extracts. The nuclear pellets obtained after the first centrifugation step were resuspended in hyper-osmotic Buffer II. After 15 min incubation on ice, nuclear debris were pelleted by centrifugation (10000 × g, 30 min) and the resulting supernatants represented the nuclear protein extracts. The cytoplasmic and nuclear extracts were stored at - 80°C.

### III - 2.2.5) Transient transfection of cells with plasmids DNA

MELN cells were transiently co-transfected with β-galactosidase reporter gene and with GSK-3β construct. Several GSK-3β constructs were analysed. GSK-3β WT (Wild Type) was the naturally occurring form of the enzyme. The constitutively active or CA form was obtained by mutation of the Ser-9 by an Ala (S9A). The mutation of Arg-96 to Ala (R96A) or Lys (R96K) abolished GSK-3β activity toward primed substrates, but not unprimed substrates (Frame *et al*., 2001). Cells were transiently transfected with plasmid DNA using the jetPEI™ reagent. This cationic polymer transfection reagent compacts DNA into positively charged particles capable of interacting with anionic proteoglycans at the cell surface and entering cells by endocytosis. This reagent also protects DNA from degradation in the cytoplasm. Following the instruction delivered by the jetPEI™ reagent company, plasmid DNA and jetPEI reagent were mixed into 150 mM NaCl and incubated at RT for 15 min. The volume of jetPEI™ added was proportional to the amount of DNA (2 µl of jetPEI™ for 1 µg of DNA). 2,8 µg of GSK-3β plasmid DNA and 0,2 µg of β-galactosidase plasmid DNA were added per well. Without changing the medium, 200 µl of jetPEI/DNA complexes in NaCl were added to each well. After 18 h incubation, the medium was replaced with new M3 medium. Then, cells were treated for 48 h with 10 nM E2 and lysates were analysed by Western blot and luciferase assay.

### III - 2.2.6) Transient transfection of cells with siRNA

MCF-7, MELN, T47D and BT-474 cells were transfected with 50 nM siRNA using Oligofectamine (6 µl per well of 6 wells/plate). Luciferase GL3 duplex siRNA was used as a control. Experiments have shown that GL3 duplex siRNA did not interfere with luciferase

expression in MELN cells. Signal Silencing GSK-3α/β siRNA were used to target both GSK-3 isoforms. GSK-3α isoform was specifically targeted by Signal Silence GSK-3α siRNA and GSK-3β isoform was specifically targeted using either siRNA duplex from Dharmacon or validated siRNA from Ambion. At 18 h post-transfection, the medium was replaced first for 24 h with M3 medium and then with M4 medium for another 24 h. After short-term (20 min) or long-term (3 h to 48 h) E2 treatment, lysis of the cells, cytoplasmic and nuclear extractions, or total RNA isolation were performed.

### III - 2.2.7) Transient transfection of cells with miR expression vectors

Isolated DNA plasmid coding for candidate miRs were transiently transfected into MCF-7 and MELN cells using FuGENE HD reagent (ratio 2:6 or 5 µg of DNA plasmid for 15 µl of FuGENE HD per well of 6 wells/plate). At 18 h post-transfection, the medium was replaced with new M3 medium and 48 h later, the cells were harvested or lysed. The transfection efficiency was checked by fluorescent microscope analysis of GFP expression (excitation: 487 nm; emission: 509 nm) in the cells. For rescue study, 5 µg of xenopus GSK-3β constructs were co-transfected with miR plasmids using 15 µl of FuGENE HD per well. Medium replacement and harvesting were performed as mentioned above.

### III - 2.2.8) Immunofluorescence analysis

MCF-7 or MELN cells were grown in M3 medium for 24 h on poly-D-lysine-coated glass coverslips and the procedures described above (treatments and transfections of cells) were followed thereafter. After the required experimental time, cells were washed with PBS and fixed for 15 min with 4 % PFA. Then, cells were washed and permeabilized for 5 min with 0,2 % Triton X-100. After washing with PBS and blocking (15 min at RT), cells were incubated (1 h at RT) with specific primary antibodies diluted in PBS + 1 % BSA. After washing, coverslips were incubated (1 h at RT) with Alexa 488-conjugated anti-mouse and/or Cy3-conjugated anti-rabbit secondary antibodies diluted in PBS + 1 % BSA. After washing, coverslips were mounted on microscope slides with elvanol. The microscope slides were stored in the dark at 4°C. For immunofluorescence microscopy, Zeiss Axioplan fluorescence microscope, Leica DFC 480 camera and Leica DFC camera (Release Notes V6.8.0) software were used. Alexa 488 and Cy3 fluorescence signals were visualized by excitation at wavelengths of 490 nm and 550 nm, respectively, using a XF2043 Dichroic Mirror.

## III - 2.3) Biochemistry

### III - 2.3.1) *In vitro* kinase assay

Commercially available rhERα turned out to be highly phosphorylated. The first step was therefore to dephosphorylate the receptor. The rhERα was incubated for 30 min at 30°C in 50 mM Tris (pH 7.5), 5 mM DTT, 2 mM $MnCl_2$ and 20 U/µg rhERα of λ-protein phosphatase. The reaction was stopped by the addition of 1 mM molybdate and 10 mM p-nitrophenyl phosphate and 10 min incubation at RT. The mix was stored at -20°C or directly used for *in vitro* kinase assay. For this reaction, a volume corresponding to 100 ng of dephosphorylated rhERα was added to 1 x GSK-3β kinase buffer and to 1 µl of recombinant rabbit GSK-3β (250000 U/ml or 50 ng/µl). After 10 min incubation at 30°C, the reaction was stopped by the addition of 1 x SDS sample buffer. The samples were subjected to SDS-PAGE and the kinase effects were detected by an antibody specific for the ERα phosphorylation site.

### III - 2.3.2) Western blot analysis

Protein concentrations of lysates, cytoplasmic and nuclear extracts were determined with the DC protein assay kit from Bio-Rad. Reading of the protein concentrations was performed in 96-well plates using the Multiskan EX plate reader. Lysates, cytoplasmic and nuclear extracts containing equal amounts of proteins (20 µg/sample) were boiled in 1 x SDS sample buffer (5 min, 95°C), subjected to 12 % SDS-PAGE and blotted on an Immobilon-P membrane. After blocking of the membrane (1 h, RT in blocking buffer), the membrane was incubated overnight at 4°C with diluted primary antibody (see table II - 6), washed and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (45 min, RT) (see table II - 7). Immunoreactive protein bands were detected with ECL-plus system. Phosphoproteins and respective proteins were detected on the same membrane after stripping (20 min, 60°C in stripping buffer). The intensities of the bands were quantified by the Image J software (NIH, USA). Results were expressed as relative phosphoprotein or protein levels standardized such that values obtained in cells treated with vehicle only were set to 1. Data represent the mean and standard errors (SEM) from a minimum of three independent experiments.

### III - 2.3.3) Ubiquitination assay

MCF-7 cells were treated with 5 µM MG132 and/or 10 nM E2 in presence or absence of siRNA targeting GSK-3α/β. Cell lysates were produced and 200 µg proteins were used for ubiquitination assay. After pre-clearing the lysates for 2 h with protein A-Sepharose beads, overnight immunoprecipitation using anti-ERα antibody and protein A-Sepharose beads was performed. Beads were centrifuged, washed and boiled in 1 x SDS sample buffer for 10 min. After centrifugation, the supernatants were processed by SDS-PAGE and western blotting. Ubiquitinated ERα was detected using anti-ubiquitin polyclonal antibody. After stripping of the membrane, immunoblotting using anti-ERα polyclonal antibody was performed as loading control.

### III - 2.3.4) Firefly luciferase reporter gene assay

MELN cells were seeded at a density of $3 \times 10^5$ cells/well in 6-well plates. After treatment for 3 h to 48 h, cells were washed with PBS, lysed (10 min, RT) with 150 µl/well of luciferase cell culture lysis reagent and collected by scraping. After centrifugation (10000 × g, 10 min), the supernatant was collected and stored at - 80°C. The luciferase activity was analyzed using the firefly luciferase assay system from Promega. 20 µl of lysate was mixed with 100µl of LAR (Luciferase Assay Reagent). This reagent contains all the substances needed for the reaction (**Fig. III - 3**), especially luciferin (substrate of the luciferase enzyme) and ATP/$Mg^{2+}$. The luminescence was immediately measured at 560 nm, RT for 1 min in a luminometer. The luminescence expressed in counts per minute (cpm) was proportional to the rate of luciferase reaction. The luciferase activity was obtained by normalizing the light intensity with the lysate protein concentration (cpm/mg protein).

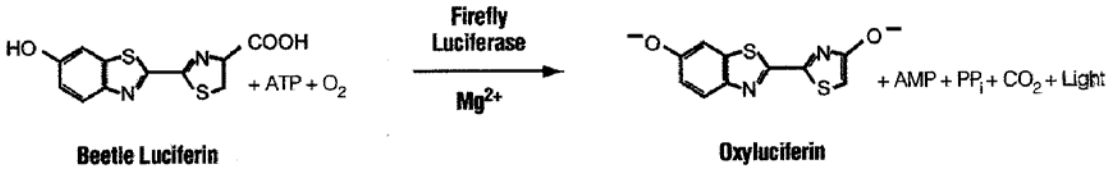


**Figure III - 3: Reaction catalysed by luciferase**. Mono-oxygenation of luciferin is catalysed by luciferase in the presence of $Mg^{2+}$, ATP and molecular oxygen. (Source: www.promega.com, Bright-Glo™ Luciferase Assay System Datasheet).

In the case of transient transfection with GSK-3β plasmid-DNA constructs, co-transfection with the reporter gene β-galactosidase was used to normalize the luciferase activity with the transfection efficiency (cpm/β-gal O.D.). β-galactosidase activity was

determined using the LacZ assay. 30 µl of protein extracts was incubated at 37°C with 450 µl of LacZ reaction buffer and 90 µl ONPG solution and after 20 min, 225 µl stop solution was added and optical density was measured with the photometer at 405 nm.

### III - 2.4) Histological methods

#### III - 2.4.1) Tissue sections

Tissue samples from breast cancer patients were obtained from collaboration with the Department of Gynecology and Obstetrics (Heidelberg University; see Schnarr *et al*., 2000). Other breast cancer samples were from collaboration with Yun Niu (Breast Cancer Pathological Department and Research Laboratory, Tianjin Cancer Hospital and Tianjin Medical University, Tianjin, China). Material was obtained with informed consent of the patients in both hospitals.

Tissues were fixed with buffered PFA, dehydrated in a graded ethanol series, embedded into paraffin and stored at RT. For further histological staining, 5 µm sections were cut from cooled (on ice) paraffin blocks using a rotation microtome and disposable blades. After a first bath in cold water, the sections were stretched in a 42°C warm bath and taken up on Histobond microscope glass slides. Then, they were dried O/N at 37°C in an incubator and stored at RT thereafter.

#### III - 2.4.2) Hematoxylin and Eosin (H & E) staining

Quality of the patients' material was checked by H & E staining. Paraffin sections were rehydrated (as described in the following paragraph), stained for 1 min with Mayer's hemalum followed by colour development in running tap water, stained for 5 min in eosin staining solution, washed and finally mounted in Kaiser's glycerol/gelatin.

#### III - 2.4.3) Immunohistochemical staining

Formalin-fixed paraffin sections were dewaxed with xylene (2 x 10 min), rehydrated with a graded series of ethanol (2 x 5 min ethanol absolute, for 2 min each 96 %, 70 % and 50 % ethanol) and sections were incubated for 5 min in distilled water. Then, the sections were submitted to heat induced antigen retrieval by microwaving in citrate buffer and humid atmosphere (water bath). The GSK-3 antigen retrieval was carried out for 2 x 5 min. After cooling down for 30 min at RT in the citrate buffer, the sections were rinsed with water (5 min), incubated with 3 % hydrogen peroxide (LSAB2 kit) for 5 min to inactivate the endogenous peroxidase, rinsed with water again and washed in PBS for 5 min. After 1 h

incubation with blocking buffer, the sections were incubated O/N at 4°C in a humid chamber with antigen specific antibody diluted in PBS containing 5 % BSA. Then, the sections were washed (3 x 5 min in PBS) and detection was performed using the manufacturer's instruction from the LSAB2 DAKO kit. The sections were incubated with a secondary biotinylated link antibody (10 min at RT), washed (3 x 5 min in PBS), incubated with Streptavidin-HRP (10 min at RT), washed (3 x 5 min in PBS) and finally stained using the DAB substrate-chromogen solution. Finally, sections were rinsed with distilled water, were counterstained with hematoxylin and mounted in Kaiser's glycerol/gelatin. For the documentation of the staining, a Zeiss Axioplan microscope equipped with a digital camera was used.

#### III - 2.4.4) Scoring of immunoreactivity

After immunohistochemical staining, the slides were examined by light microscopy and were scored together by two observers (D. Mayer and J. Grisouard). GSK-3β immunostaining was mainly cytoplasmic and overall constant for each tissue slide. Therefore, the slides were scored as negative/faint (0), very weak to weak (1) and moderate to strong (2) according to the cytoplasmic intensity of the staining. In few rare cases, only parts of the tissue sections were stained or nuclear staining was observed.

### III - 2.5) Statistical Analysis

For quantitative analysis of western blots, signal intensities were determined with the Image J software and were normalized with β-actin used as loading control. Luciferase analysis was performed using Berthold LB 9505 C (version 4.08) software. For quantitative PCR, MJ opticon monitor analysis software (version 3.1) from Bio-Rad was used and loading variations were normalized by β-actin. For each set of data, mean ± SEM was calculated evaluating three independent experiments. Differences between groups were statistically evaluated using *t*-test. A $P$ value $< 0.05$ was considered significant.

Data obtained from immunohistochemical staining of patients' tissue specimens were statistically evaluated using MedCalc statistical software and Fisher's exact test was applied to reveal differences between the observed proportions of two independent samples. A $P$ value $< 0.05$ was considered significant.

# IV - Results

Previous work using confocal microscopy showed a co-localisation of ERα and GSK-3β in MCF-7 breast cancer cells. Co-localisation was observed in the cytoplasm in resting cells and in the nucleus in estradiol-treated cells. These results suggested a functional correlation of the two proteins (Medunjanin *et al*., 2005) and a possible role of 17β-estradiol (E2) both in the sub-cellular localisation of these proteins and in the regulation of the ERα/GSK3 interaction. The present work unravels the functional relationship of GSK-3 and ERα and the role of E2 therein.

## IV - 1) Effects of E2 on ERα and GSK-3α/β cellular localisation and phosphorylation

### IV - 1.1) E2 induced nuclear translocation of ERα but did not alter GSK-3α/β cellular localisation

First, the sub-cellular localisation status of GSK-3 and ERα were studied by immunofluorescence imaging in MCF-7 cells treated or not with E2 (**Fig. IV - 1**). In untreated cells, ERα and GSK-3 were localised both in the cytoplasm and in the nucleus of the cells. An increase of the nuclear staining of ERα was observed after E2 treatment suggesting an E2-induced translocation of the receptor upon ligand binding. However, GSK-3 sub-cellular protein content and distribution did not seem to be altered upon E2 stimulation.

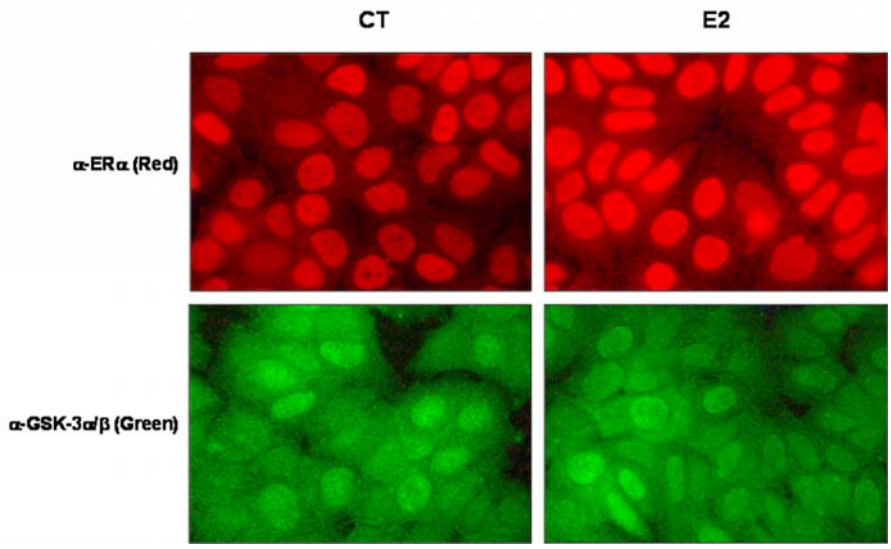


**Figure IV - 1: Effects of E2 treatment on cellular localisation of ERα and GSK-3α/β.** MCF-7 cells were left untreated (CT) or were treated with 100 nM E2 for 20 min (E2). Immunofluorescence staining of ERα protein (red) and GSK-3α/β (green) shows E2-dependent increase of nuclear ERα, whereas GSK-3α/β cellular localization did not seem to be altered by E2 treatment.

### IV - 1.2) E2-induced phosphorylation of ERα at Ser-118 and E2-related phosphorylation of GSK-3α/β at Ser-21/9

To study the time-dependent effects of E2 on ERα and GSK-3 function in MCF-7 cells, western blot analysis was performed on cell lysates from E2-treated cells using phospho- and protein-specific antibodies. The phosphorylation status of ERα and GSK3 indicates the level of activation or inhibition of these proteins, respectively. Indeed, E2-induced phosphorylation of ERα at Ser-118 is crucial for full activation of the receptor (Lannigan *et al.*, 2003), whereas phosphorylation of GSK-3α and GSK-3β at Ser-21 and Ser-9, respectively, inhibits the kinase activities.

After treatment of MCF-7 cells with 100 nM E2 for 5 to 60 min, ERα protein content in cell lysates was not altered indicating that this protein was stable during 60 min of E2 treatment (**Fig. IV - 2**). From 0 to 30 min of E2 exposure, ERα phosphorylation at Ser-118 increased progressively and a plateau was reached thereafter. Regarding GSK-3α/β, the protein contents were not altered by E2 treatment of the cells and progressive phosphorylation of both kinase isoforms was observed upon E2 stimulation (**Fig. IV - 2**). GSK-3α phosphorylation signal at Ser-21 (51 kDa band) was weaker than GSK-3β phosphorylation signal at Ser-9 (47 kDa band). This result may be explained by the more prominent expression of the GSK-3β isoform in comparison with GSK-3α isoform (47 kDa GSK-3β versus 51 kDa GSK-3α bands after GSK-3α/β immunostaining).

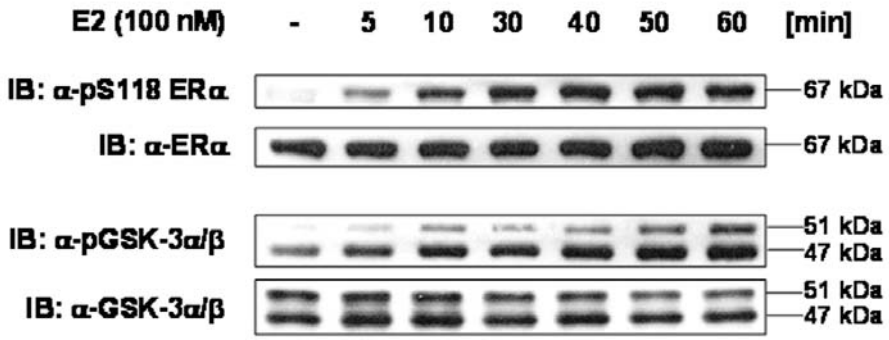


**Figure IV - 2: E2-induced phosphorylation of ERα at Ser-118 and E2-related phosphorylation of GSK-3α/β at Ser-21/9.** Cells were left untreated (-) or were treated with 100 nM E2 from 5 min to 60 min. After lysis of the cells, immunoblot (IB) analysis showed that E2 time-dependent exposure increases both Ser-118 phosphorylation of ERα and Ser-21/9 phosphorylation of GSK-3α/β. Immunodetection of ERα and GSK-3α/β proteins was used as respective loading control.

### IV - 1.3) E2-induced nuclear phosphorylation of ERα and E2-related cytoplasmic phosphorylation of GSK-3α/β

Specific co-immunofluorescence staining (yellow) of ERα (red) and Ser-118 phosphorylation at the ERα (green) revealed the appearance of nuclear speckles representing phosphorylated receptors after 5 min of E2 stimulation (**Fig. IV – 3A**). With increased E2 exposure time, cytoplasmic ERα content seemed to decrease and nuclear accumulation of the receptor seemed to be enhanced. Furthermore, a transient ERα accumulation at the plasma membrane was observed at 20 min of E2 exposure. At this time point of E2 stimulation, the speckled phospho-receptor pattern was not observed anymore but the nuclei of the cells were stained in yellow suggesting an increase of Ser-118 phosphorylated ERα. At 60 min of E2 exposure, the intensity of the yellow colour was even more pronounced. In addition, cellular fractionation followed by western blot analysis (**Fig. IV - 4**) demonstrated more quantitatively the ERα translocation from the cytoplasm to the nucleus upon E2 stimulation. The longer the cells were stimulated with E2, the less ERα protein was in the cytoplasm and the more ERα protein was in the nucleus. Phosphorylation of the receptor at Ser-118 was only observed in the nucleus and increased with the time of E2 exposure. These data confirmed the E2-induced Ser-118 phosphorylation pattern (**Fig. IV - 2**) and the co-immunofluorescence data (**Fig. IV - 3A**) previously described.

Analysis of GSK-3 proteins (green) and their phosphorylated forms (red) by co-immunofluorescence suggested that GSK-3 proteins were present both in the cytoplasm and nucleus of MCF-7 cells and the phosphorylation of the kinases was mainly cytoplasmic and more specifically located in the peri-nuclear region of the cells (**Fig. IV – 3B**). Additionally, these data suggest that E2-related phosphorylation of GSK-3 reached a maximum at 20 min of E2 treatment. The cellular fractionation did not show any clear E2-induced alteration between the cytoplasmic and nuclear GSK-3 protein levels (**Fig. IV - 4**). Moreover, the cellular fractionation confirmed the E2-induced increase of the GSK-3β phosphorylation at Ser-9 and confirmed that this phosphorylation was mainly located in the cytoplasm of the cells. However, E2-related GSK-3α phosphorylation seen in cell lysates (see **Fig. IV - 2**) was hardly observed after cellular fractionation. This may be explained by the fact that GSK-3α isoform protein level was lower than GSK-3β isoform protein level.

In conclusion, E2 induced ERα translocation into the nucleus, where phosphorylation of the receptor at Ser-118 occurred suggesting a role for phosphorylation of this serine residue

in the nuclear activation of the receptor. Moreover, E2-related phosphorylation of GSK-3, and especially the GSK-3β isoform, was observed in the cytoplasm and strategically located at the peri-nuclear site suggesting that GSK-3 is a gatekeeper at the nuclear membrane and may be involved in the regulation of ERα translocation from the cytoplasm to the nucleus.

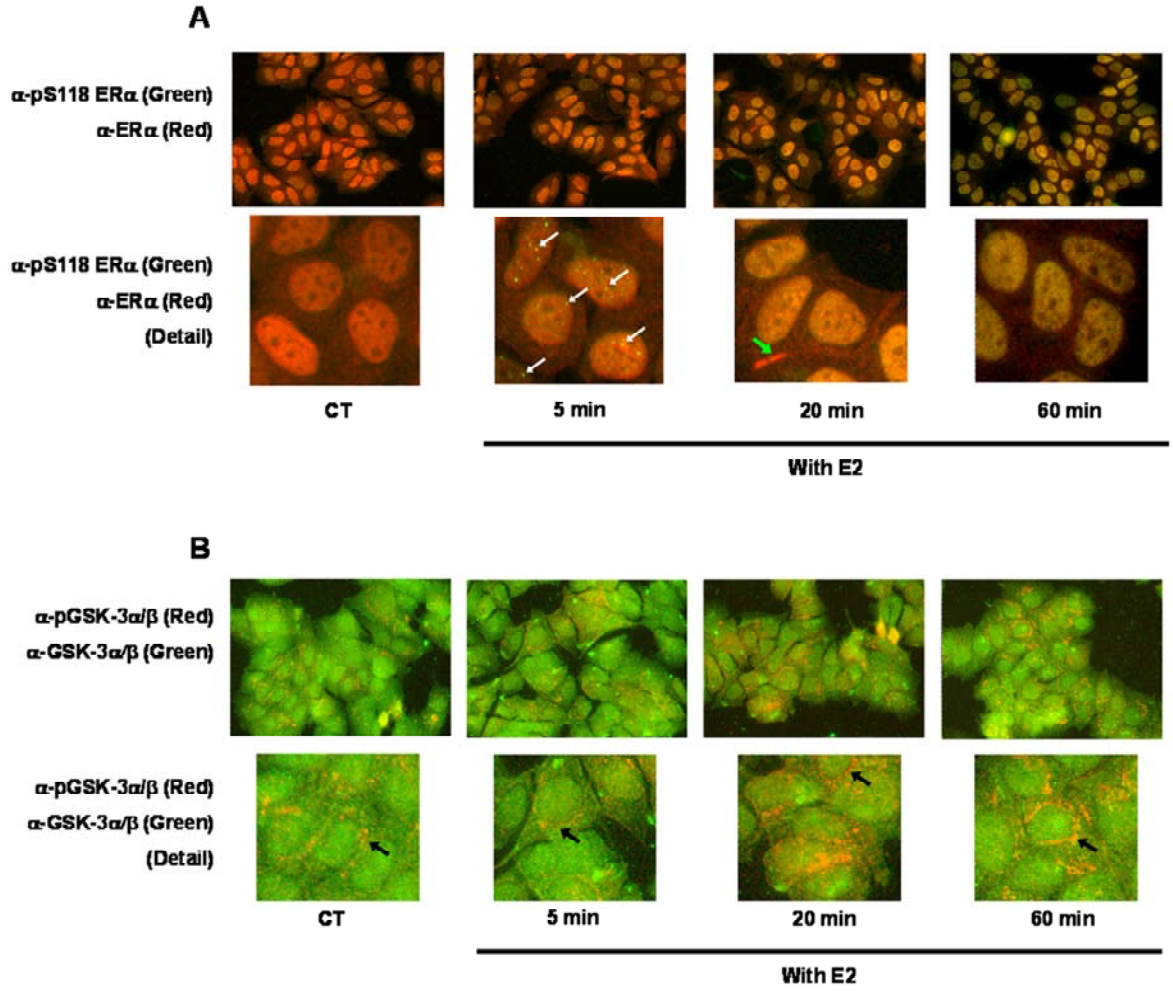


**Figure IV - 3: Effects of E2 treatment on cellular localisation and phosphorylation of ERα and GSK-3α/β.** MCF-7 cells were left untreated (CT) or were treated with 100 nM E2 for 5 min, 20 min and 60 min. ***Panel A.*** Immunofluorescence staining of ERα protein (red) and pSer-118 ERα (green) shows E2 time-dependent increase of nuclear co-localization (yellow) resulting from close association of red and green fluorescence. Transient events were also observed. At 5 min of E2 treatment, a speckled pattern of Ser-118 phosphorylation of nuclear ERα was observed (white arrows). At 20 min of E2 treatment, membrane accumulation of ERα was detected (green arrow). ***Panel B.*** Immunofluorescence staining of GSK-3α/β protein (green) and pSer-21/9 GSK-3α/β (red) shows peri-nuclear co-localization (black arrows) resulting from close association of red and green fluorescence (yellow). At 20 min E2 treatment, phosphorylation of GSK-3α/β seemed to reach a maximum (orange colour of co-localization) and was localized mainly throughout the cellular cytoplasm. At 60 min, a strong co-localization signal was still observed and it was more distinctly located in the peri-nuclear region of the cells.

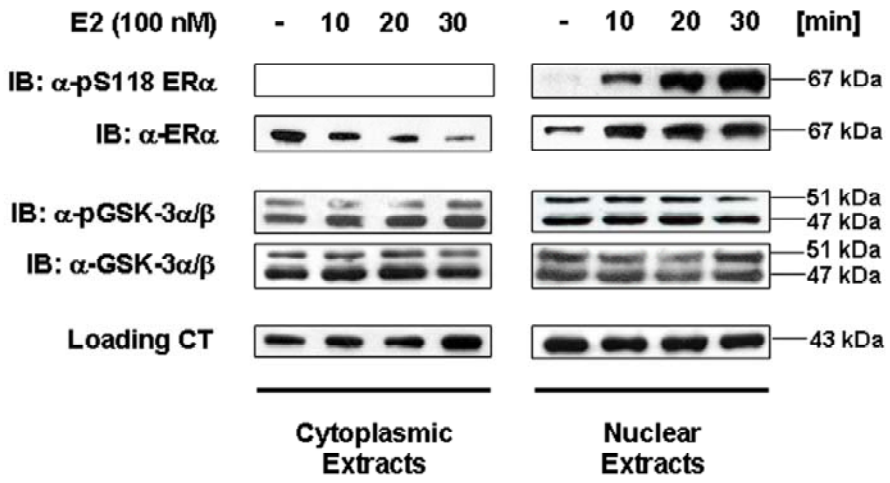


**Figure IV - 4: E2-induced ERα translocation accompanied by nuclear phosphorylation of the receptor at Ser118 and E2-related cytoplasmic phosphorylation of GSK-3α/β at Ser-21/9.** Cells were left untreated (-) or were treated with 100 nM E2 from 10 min to 30 min. Cellular fractionation was performed and immunoblots (IB) show E2 time-dependent increase of both ERα translocation into the nucleus and nuclear ERα phosphorylation at Ser-118 as well as E2 time-dependent exposure increase of cytoplasmic phosphorylation of GSK-3α/β at Ser-21/9. β-actin and replication protein A / p34 were used as loading controls for cytoplasmic and nuclear extracts, respectively.

## IV - 2) GSK-3β phosphorylated ERα at Ser118 *in vitro*

Since Ser-118 phosphorylation is an important step in ERα activation we investigated if this site is a target for GSK-3. In order to demonstrate that GSK-3 phosphorylates ERα, radioactive *in vitro* kinase assay was performed, which showed an increase of recombinant human ERα (rhERα) phosphorylation in presence of rabbit GSK-3β (Medunjanin *et al*., 2005). In addition, *in vitro* kinase assay using wild type and mutant (Ser- to Ala-) GST-ERα fusion proteins were performed to identify Ser-118 ERα residues as GSK-3β phosphorylation site (Medunjanin *et al*., 2005). These results were confirmed by *in vitro* kinase assay and immunoblotting. Using rhERα, GSK-3β and an antibody detecting specifically phosphorylation of ERα at Ser-118, we demonstrated that ERα can be phosphorylated by GSK-3β at Ser-118 (**Fig. IV - 5**). Overall, these results suggest that GSK-3 may play a role in ERα activation.

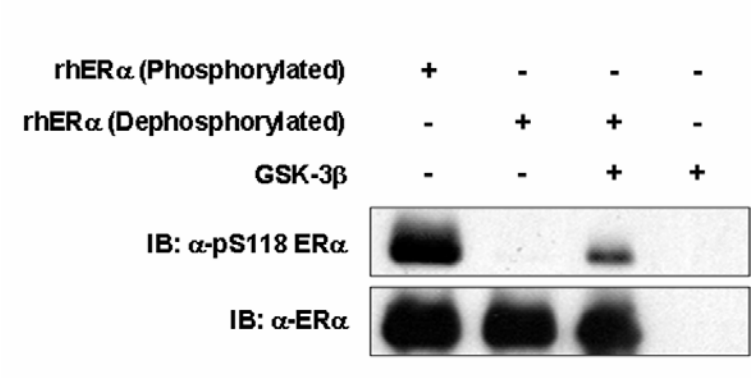


**Figure IV - 5: GSK-3 phosphorylates ERα at Ser-118 *in vitro*.** Kinase assay using dephosphorylated recombinant human ERα (rhERα) and purified GSK-3β followed by immunoblotting (IB) with pSer-118 phosphorylation-site specific antibody for ERα shows that GSK-3β phosphorylates ERα at Ser-118.

## IV - 3) Effect of GSK-3β inhibitors on ERα signalling pathway and on MCF-7 cell proliferation

Next, in order to unravel the role of GSK-3 regarding E2 signalling pathway and to investigate the role of GSK-3 on E2-induced ERα-positive breast cancer cell proliferation, the effect of GSK-3β inhibitors on ERα phosphorylation and intracellular localisation as well as on E2-induced MCF-7 cells proliferation was studied.

### IV - 3.1) LiCl-inhibits E2-induced ERα phosphorylation at Ser-118 and maleimide inhibitors are inefficient regarding this signalling pathway

MCF-7 cells were treated for 20 min with 100 nM E2 in presence or absence of LiCl and maleimide inhibitors (SB415286 and SB216763). After cell fractionation, the effects of these ATP/$Mg^{2+}$ competitive inhibitors of GSK-3 kinase were studied on cytoplasmic and nuclear extracts used for immunoblot analysis (**Fig. IV - 6A-B**).

In accordance with the part IV - 1, E2 triggered ERα translocation from the cytoplasm to the nucleus, where phosphorylation at Ser-118 occurred (**Fig. IV - 6A-B**). LiCl alone did not show any effects regarding Ser-118 phosphorylation of ERα and the intracellular localisation of the receptor. However, LiCl decreased E2-induced nuclear ERα phosphorylation at Ser-118 without any change regarding E2-induced ERα translocation into the nucleus. For SB216763 alone, ERα protein content in each extracts and nuclear Ser-118 phosphorylation were similar to the control. Interestingly, an increase of ERα translocation

and Ser-118 phosphorylation were observed with SB415286 treatment alone. Moreover, none of the maleimide inhibitors altered E2-induced translocation of the ERα into the nucleus and phosphorylation at Ser-118. However, after E2 treatment and in comparison with LiCl, a stronger ERα cytoplasmic decrease was observed in presence of these maleimide inhibitors.

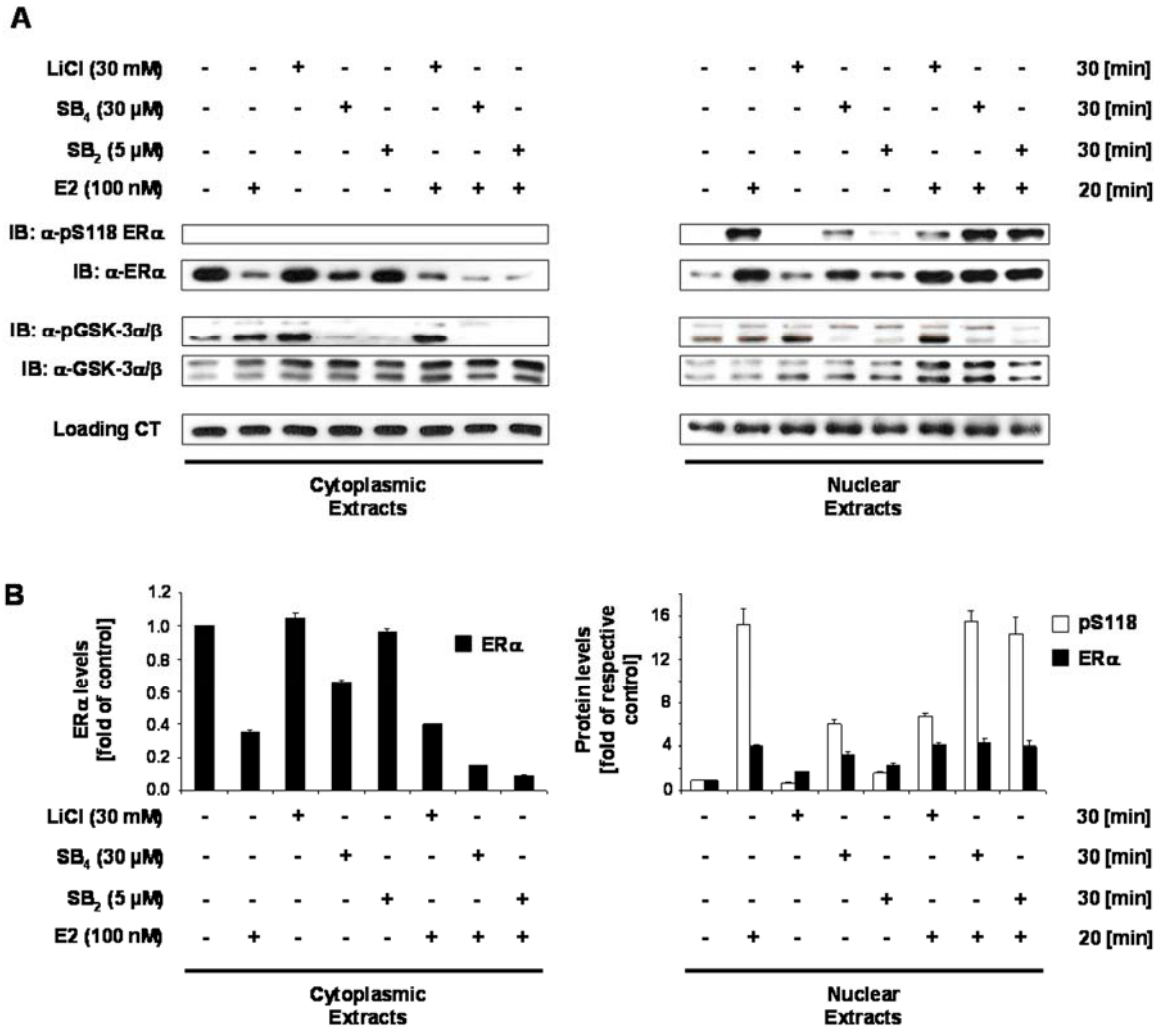


**Figure IV - 6: Effect of GSK-3 inhibitors on ERα phosphorylation and intracellular localisation.** Cells were left untreated or were treated with either 30 mM LiCl or 30 µM SB415286 ($SB_4$) or 5 µM SB216763 ($SB_2$) alone for 30 min. When cells were treated with both GSK-3 inhibitors and E2, cells were first pre-treated with inhibitors for 10 min and then treated with 100 nM E2 for 20 min. After treatment, cellular fractionation was performed. ***Panel A.*** Immunoblots (IB) show decrease of E2-induced nuclear ERα phosphorylation at Ser-118 by LiCl treatment whereas the maleimide inhibitors $SB_4$ and $SB_2$ did not alter this phosphorylation. Upon E2 treatment, GSK-3α/β phosphorylation status at Ser-21/9 increased in the cytoplasm of the treated cells. Both in absence and presence of E2, LiCl increased cytoplasmic and nuclear GSK-3β phosphorylation whereas maleimide inhibitors decreased GSK-3β phosphorylation in both cellular compartments. β-actin and replication protein A / p34 were used as loading controls for cytoplasmic and nuclear extracts, respectively. ***Panel B, left.*** Quantitative analysis of cytoplasmic ERα protein level given as fold of control and corrected for potential loading variations using β-actin. ***Panel B, right.*** Quantitative analysis of nuclear ERα protein level and pS118 ERα signal intensity given as fold of control and corrected for potential loading variations using replication protein A / p34. Three independent experiments were analyzed.

E2-related GSK-3β phosphorylation at Ser-9 was observed in the cytoplasmic fraction and no obvious effects of E2 on GSK-3α/β intracellular distribution was noticed (**Fig. IV - 6A**). After LiCl treatment (with or without E2 treatment), a strong signal was obtained for GSK-3β phosphorylation at Ser-9 in both cytoplasmic and nuclear extracts. At the opposite, GSK-3α or β were not phosphorylated after maleimide treatments, neither in the cytoplasm, nor in the nucleus. The GSK-3 protein contents were difficult to analyse from these blots and variations of signal, especially in the nuclear extracts may be explained by loading discrepancies.

Overall, LiCl inhibited kinase activity of GSK-3 by phosphorylation of GSK-3β at Ser-9, while maleimide inhibitors markedly decreased the phosphorylation status of GSK-3α/β. None of the GSK-3 inhibitors altered E2-induced ERα translocation and LiCl was the only studied inhibitor to decrease E2-induced ERα phosphorylation at Ser-118. Finally, SB415286 alone induced ERα translocation and Ser-118 phosphorylation suggesting estrogenic effects of this inhibitor.

### IV - 3.2) GSK-3β inhibitors blocked MCF-7 cells proliferation

MCF-7 cells growth curves were obtained by counting of the cells after 24 h, 48 h and 72 h treatment (**Fig. IV - 7**). Basal growth rate was determined by counting untreated cells. E2 increased the growth rate. LiCl and maleimide inhibitors inhibited basal and E2-induced proliferation of the MCF-7 cells suggesting that GSK-3 plays a role in MCF-7 cell proliferation. However, the molecular mechanisms affected by GSK-3 inhibitors to inhibit MCF-7 cell proliferation are unclear and the hypothetical effects of GSK-3 on the E2 signalling pathway can not be solved by this approach.

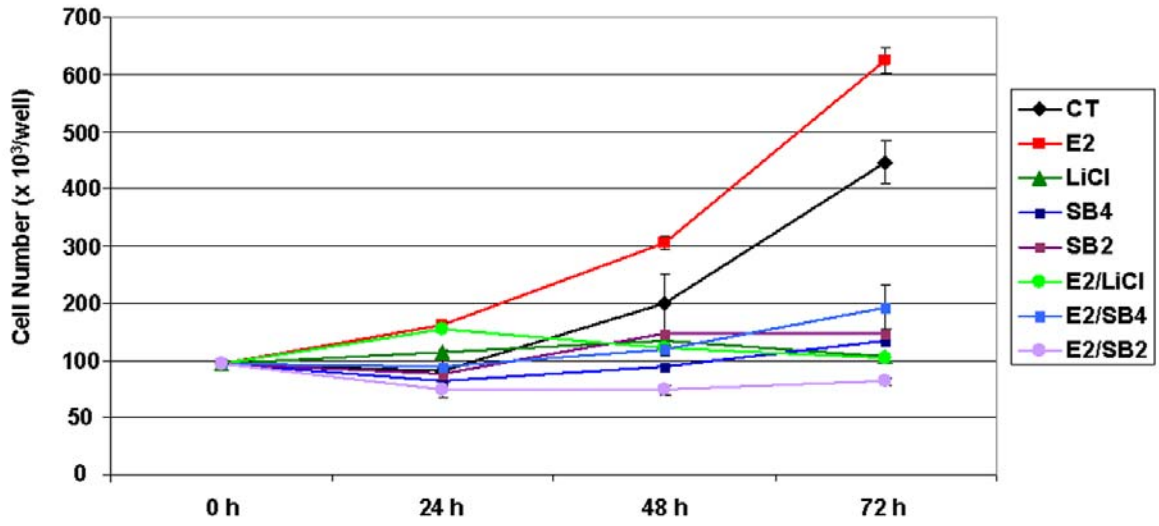


**Figure IV - 7: GSK-3β inhibitors blocked MCF-7 cells proliferation.** MCF-7 cells were grown in DMEM supplemented with 10 % DCC-FCS and remained untreated (CT) or were treated with E2 (100 nM), LiCl (30 mM), $SB_4$ (30 µM), $SB_2$ (5 µM) and combinations thereof. Cells were counted at 24 h intervals and the number of seeded cells was taken as reference (0 h time point). E2-induced cell proliferation (red curve) and basal cell proliferation (black curve) were inhibited by the use of GSK-3β inhibitors (green, blue and purple curves for LiCl, $SB_4$ and $SB_2$, respectively).

## IV - 4) Silencing of GSK-3 and its consequences on ERα signalling pathway in ERα-positive human breast carcinoma cell lines

RNA interference (RNAi) using small interfering RNA (siRNA) targeting GSK-3 was used to specifically silence GSK-3 protein expression in order to investigate the involvement of GSK-3 in ERα function and activity.

### IV - 4.1) GSK-3α/β silencing causes decrease of ERα protein content

MCF-7 cells were transfected with siRNA targeting GSK-3α/β in order to silence GSK-3 expression. After transfection with siRNA, GSK-3α and GSK-3β protein levels were reduced by 60 % to 70 % in comparison with the untransfected and control siRNA-transfected cells (**Fig. IV - 8A-B**). The addition of 100 nM E2 for 48 h did not affect the silencing of GSK-3α/β.

E2 treatment of untransfected cells for 48 h resulted in an approximately 50 % decrease of ERα protein level (**Fig. IV - 8C-D**) which agreed with previous reports that prolonged treatment with E2 downregulates the steady-state level of ERα in MCF-7 cells (Saceda *et al.*, 1988; Laios *et al.*, 2005). In cells not treated with E2, silencing of GSK-3α/β

caused a significant decrease of ERα protein level (approximately 35 %). Combination of GSK-3α/β silencing and treatment with 100 nM E2 for 48 h, led to an even more pronounced and significant ERα downregulation (approximately 90 %) in MCF-7 cells.

Similar results as described for MCF-7 cells were also observed in T47D and BT-474 ERα-positive human breast carcinoma cells (**Fig. IV - 9**). In both cell lines, GSK-3α/β silencing led to a significant reduction of ERα protein levels.

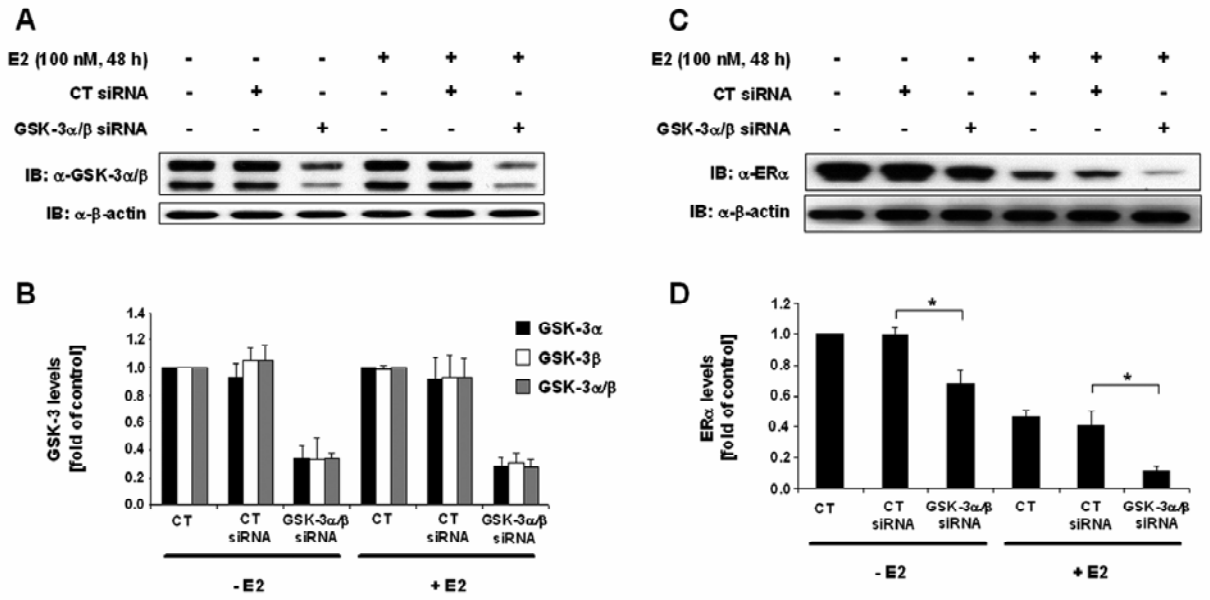


**Figure IV - 8: Decrease of ERα protein in MCF-7 cells after GSK-3α/β silencing.** Cells were left untransfected (CT) or were transfected either with 50 nM GL3 control siRNA (CT siRNA) or with 50 nM siRNA targeting GSK-3α/β (GSK-3α/β siRNA); then the cells were treated or not with 100 nM E2 for 48 h. ***Panel A.*** Immunoblot (IB) showing silencing of GSK-3α/β. β-actin was used as loading control. ***Panel B.*** Quantitative analysis of GSK-3α (black), GSK-3β (white) or both isoforms (grey) from three independent RNAi experiments. ***Panel C.*** Immunoblot showing downregulation of ERα protein after GSK-3α/β silencing. ***Panel D.*** Quantitative analysis of ERα protein level given as fold of control and corrected for potential loading variations using β-actin. Three independent experiments were analyzed and error bars represent the standard error of the mean (*, $p < 0.05$ determined by *t*-test).

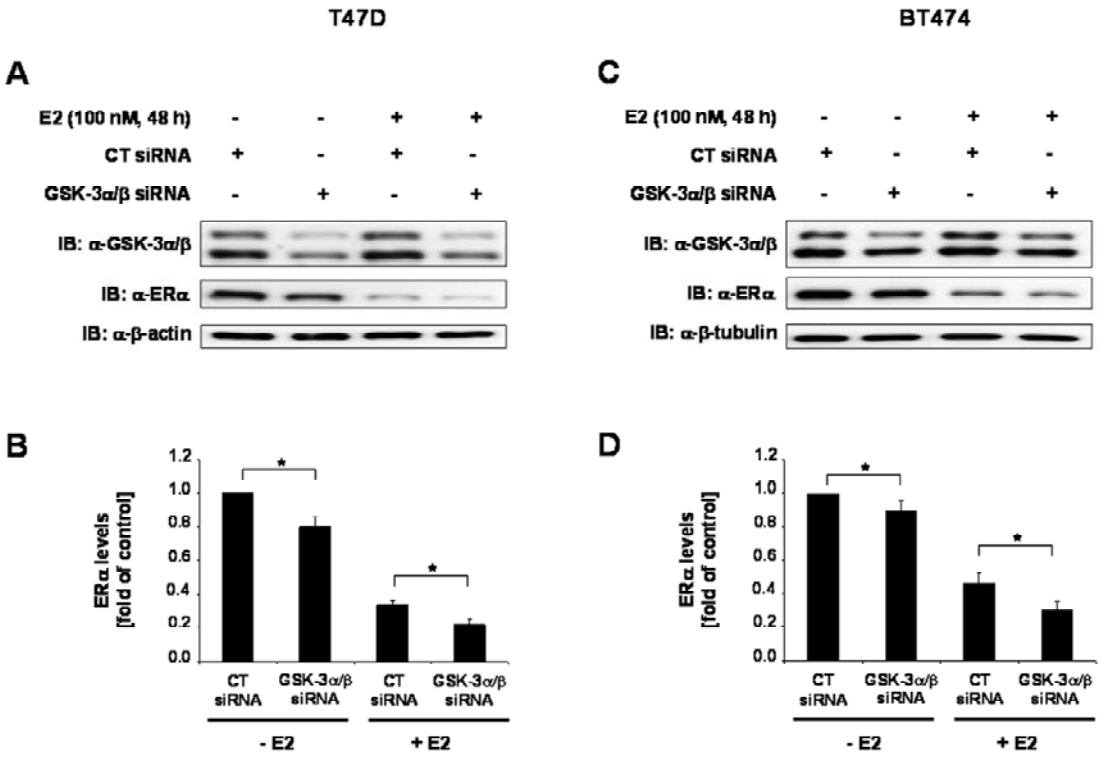


**Figure IV - 9: Decrease of ERα protein in T47D and BT-474 cells after GSK-3α/β silencing.** Cells were transfected either with 50 nM GL3 control siRNA (CT siRNA) or with 50 nM siRNA targeting GSK-3α/β (GSK-3α/β siRNA) and treated or not with 100 nM E2 for 48 h. Immunoblots (IB) show down-regulation of ERα protein after GSK-3α/β silencing in T47D cells (***Panel A***) and BT-474 cells (***Panel C***). Quantitative analysis in T47D cells (***Panel B***) and BT-474 (***Panel D***) of ERα protein level given as fold of control and corrected for potential loading variations using β-actin and β-tubulin, respectively. Three independent experiments were analyzed and error bars represent the standard error of the mean (*, $p < 0.05$ determined by *t*-test).

### IV - 4.2) Effect of GSK-3 silencing on ERα mRNA expression

GSK-3 silencing-related decrease of ERα protein level described above might be due to a decrease of ERα gene transcription leading to downregulation of ERα biosynthesis. To investigate the hypothetical regulation of ERα transcription by GSK-3, quantitative real-time PCR experiments were performed using ERα primers with cDNA synthesized from RNA extracted from MCF-7 cells that had been transfected or not with GSK-3α/β siRNA and treated or not with 10 nM E2 for 6 h, 24 h, or 48 h (**Fig. IV - 10**). Basal ERα mRNA expression in the GSK-3α/β siRNA-transfected cells was significantly decreased at the 6 h time point compared to control siRNA-transfected cells. However, basal ERα mRNA expression was similar for the control siRNA and GSK-3α/β siRNA transfected cells at the 24 h and the 48 h time points. It has been reported that E2 treatment induces decrease of ERα mRNA expression in MCF-7 cells (Saceda *et al*., 1988; Read *et al.*, 1989; Berthois *et al*.,

1990; Pink and Jordan, 1996). In our experiments E2-related decrease of ERα mRNA expression was confirmed and was similar (about 50 to 60 %) in control siRNA and GSK-3α/β siRNA transfected cells at all time points studied. After 48 h E2-treatment the decrease of ERα mRNA expression was slightly stronger upon GSK-3 silencing, this was, however, not statistically significant. Taken together, the results show that GSK-3 silencing has a transient effect on basal ERα mRNA expression level whereas GSK-3 silencing does not significantly alter E2-related decrease of ERα mRNA levels.

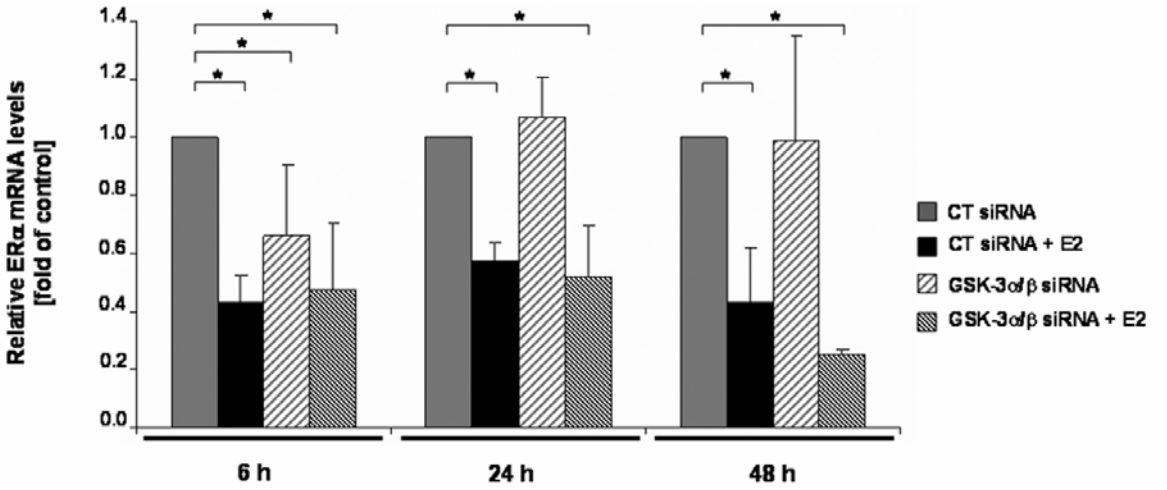


**Figure IV - 10: Effect of GSK-3α/β silencing on ERα mRNA expression in MCF-7 cells.** Cells were transfected either with GL3 control siRNA (CT siRNA) or with siRNA targeting GSK-3α/β (GSK-3α/β siRNA) and treated or not with 10 nM E2 for 6 h, 24 h and 48 h. Total RNA was extracted and used to synthesize cDNA by reverse transcription. Quantitative real- time PCR using ERα primers showed that E2-related decrease of ERα mRNA expression was not significantly altered upon GSK-3α/β silencing. β-actin was used as an internal control. Three independent experiments were analyzed and error bars represent the standard error of the mean (*, $p < 0.05$ determined by *t*-test).

### IV - 4.3) Increase of ERα proteasomal degradation rather than reduction of ERα protein synthesis downregulates ERα protein upon GSK-3α/β silencing

The results described above raise the question on the mechanism leading to ERα protein decrease in GSK-3α/β silenced cells. Experiments using the translation inhibitor cycloheximide (CHX) and the proteasome inhibitor MG132 were performed to investigate the role of GSK-3 regarding ERα protein synthesis and ERα proteasomal degradation, respectively (**Fig. IV - 11**).

In cells transfected with control siRNA (**Fig. IV - 11, left panel**), CHX slightly decreased ERα protein content whereas MG132 slightly increased ERα protein content. In

cells transfected with GSK-3α/β siRNA and not stimulated with E2, the proteasome inhibitor MG132 significantly rescued GSK-3α/β silencing related-ERα protein decrease. Such significant rescue was also observed when MG132 was used in combination with CHX. In E2 treated cells (**Fig. IV - 11, right panel**), MG132 treatment rescued E2 induced- as well as GSK-3α/β silencing related-ERα protein decrease. In GSK-3α/β silenced cells treated with both CHX and E2, a significant MG132 mediated rescue of ERα was still observed. Taken together, these results suggest that downregulation of ERα upon GSK-3α/β silencing either in unstimulated or in E2 treated cells is due to increase of ERα proteasomal degradation rather than alteration of ERα protein synthesis.

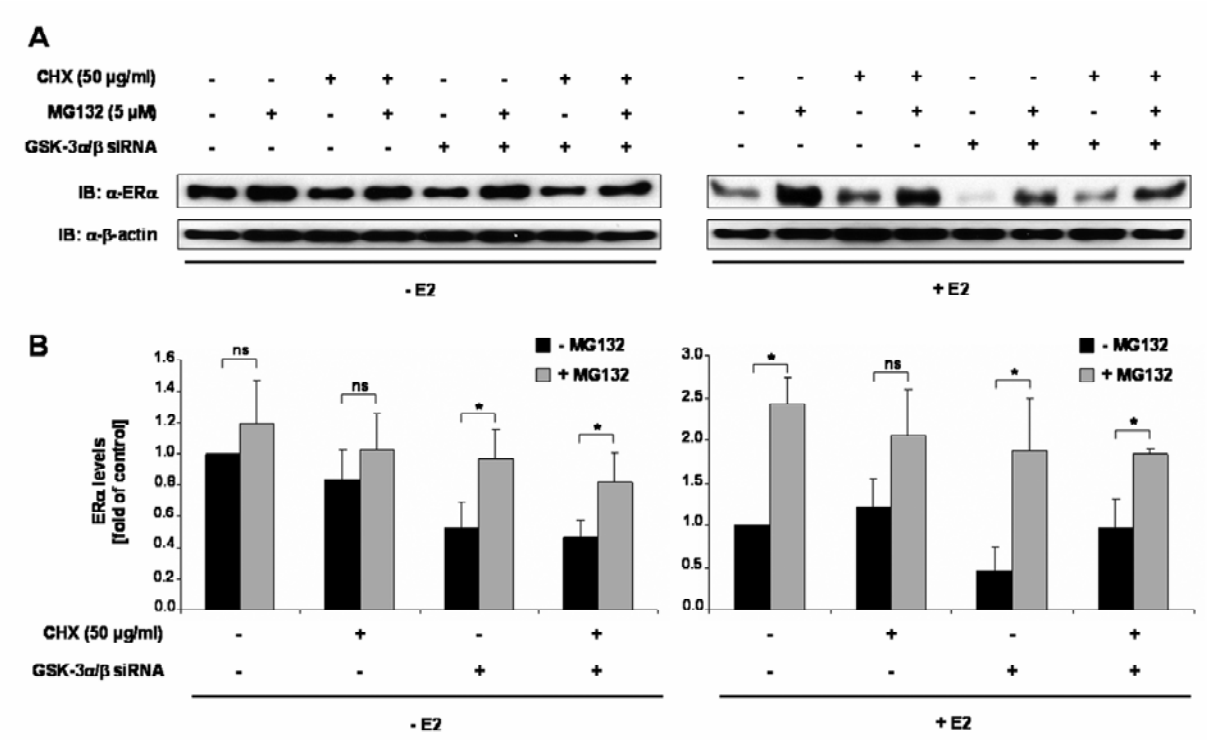


**Figure IV - 11: ERα protein decrease caused by GSK-3α/β silencing is due to an increase of proteasomal degradation rather than decreased ERα protein synthesis.** Cells were transfected with CT siRNA or with siRNA targeting GSK-3α/β (GSK-3α/β siRNA). After 1 h pre-treatment with either 5 µM MG132 or 50 µg/ml cycloheximide (CHX) or a combination of both, cells were treated **(right panel)** or not **(left panel)** with 10 nM E2 for 6 h. ***Panel A.*** Immunoblots (IB) showing rescue of GSK-3α/β silencing-related ERα protein decrease by inhibition of proteasomal degradation even where translation was blocked with CHX. β-actin was used as loading control. ***Panel B.*** Quantitative analysis of ERα protein level given as fold of control and corrected for potential loading variations using β-actin. Three independent experiments were analyzed and error bars represent the standard error of the mean (*, $p < 0.05$ determined by *t*-test).

### IV - 4.4) GSK-3 prevents ERα ubiquitination and proteasomal degradation

Several studies recently described the involvement of the ubiquitin-proteasome pathway in the E2-induced degradation of ERα (Nawaz *et al*., 1999; Lonard *et al*, 2000; Preisler-Mashek *et al*., 2002). From the results shown in **Fig. IV - 8**, we hypothesized that GSK-3α/β may be involved in ERα stabilization, preventing its ubiquitination and proteasomal degradation.

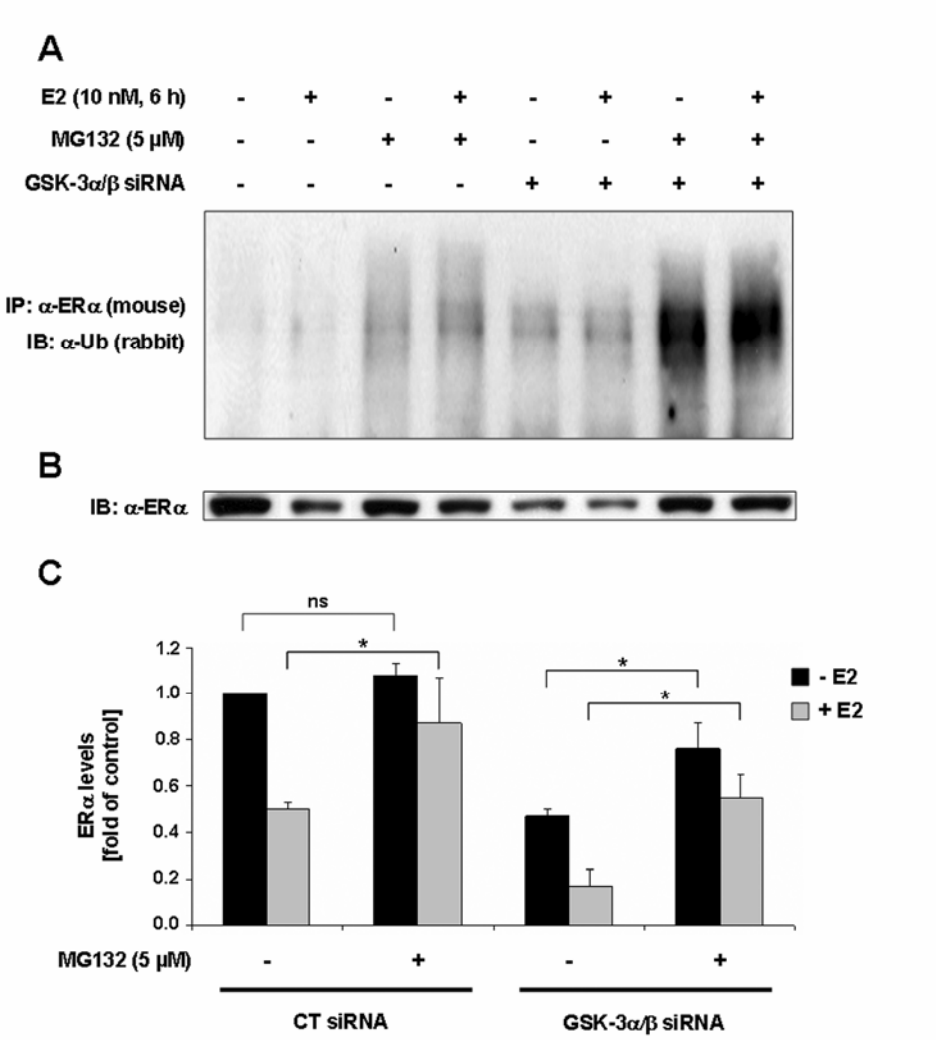


**Figure IV - 12: ERα ubiquitination and proteasomal degradation is increased upon GSK-3 silencing.** Cells were transfected either with GL3 control siRNA (CT siRNA) or with siRNA targeting GSK-3α/β (GSK-3α/β siRNA). After 1 h pre-treatment with 5 µM MG132 where indicated, cells were treated or not with 10 nM E2 for 6 h. ***Panel A.*** ERα immunoprecipitates (IP) from MCF-7 cells were subjected to immunoblotting (IB) detecting ubiquitinated forms of ERα which were found strongly increased upon silencing of GSK-3α/β. ***Panel B.*** Detection of ERα on the same membrane shown in panel *A*. ***Panel C.*** Quantification of ERα protein levels given as fold of control from three independent experiments including that shown in panel B and error bars represent the standard error of the mean (*, $p < 0.05$ determined by *t*-test; ns, not significant).

To analyze this, ERα was immunoprecipitated from cells transfected with control or GSK-3α/β siRNA followed by immunoblotting with anti-ubiquitin antibody (**Fig. IV - 12A**). After GSK-3α/β silencing, we detected an increase in ubiquitination of ERα in cells treated or not with E2 compared to the control siRNA-transfected cells. When proteasomal degradation was inhibited using MG132, further accumulation of ubiquitinated forms of ERα was observed. Detection of ERα protein in these samples confirmed ERα downregulation caused either by E2 treatment or by GSK-3α/β silencing (**Fig. IV - 12B-C**). Addition of MG132 rescued the downregulation of ERα caused by E2 in control siRNA-transfected cells and, more importantly, in cells after GSK-3α/β silencing both in presence or absence of E2. These results suggest an important role of GSK-3 regarding ERα stabilization.

**IV - 4.5) ERα is a substrate for GSK-3 and is phosphorylated at Ser-118**

In **Fig. IV - 5**, it was shown that GSK-3 phosphorylated ERα at Ser-118 in an *in vitro* kinase assay. In order to investigate if ERα is a substrate for GSK-3 *in vivo*, we further analyzed the effects of GSK-3α/β silencing on the phosphorylation of ERα at Ser-118 induced by 100 nM E2 stimulation for 20 min. In untransfected and control siRNA-transfected cells, short-term E2 treatment induced ERα phosphorylation at Ser-118 (**Fig. IV - 13A-B**). This phosphorylation was significantly reduced after GSK-3α/β silencing. Furthermore, in accordance with our results shown in **Fig. IV - 8**, ERα protein content was reduced in these cells (**Fig. IV - 13A-B**). In addition, a clear although not significant decrease of the pS118/ERα ratio was observed (**Fig. IV - 13B**) suggesting that ERα downregulation was not the only reason for the reduction of ERα phosphorylation level at Ser-118.

In order to further clarify the role of GSK-3 in ERα phosphorylation *in vivo*, we investigated Ser-118 phosphorylation of ERα after inhibition of the proteasome. Under this condition, we confirmed that ERα degradation due to GSK-3α/β silencing was partly prevented by MG132 addition (**Fig. IV - 13C-D**, **right part**). Importantly, ERα protein rescue was not accompanied by a rescue of E2-induced Ser-118 phosphorylation in GSK-3α/β silenced cells (**Fig. IV - 13C**), which is also evident from the reduced pS118 / ERα ratio (**Fig. IV - 13D**). These results provide further evidence that Ser-118 of ERα is a target for E2-induced phosphorylation by GSK-3α/β in MCF-7 cells. Interestingly, E2-induced phosphorylation of ERα at Ser-118 was also decreased in MG132-treated cells

transfected with control siRNA showing that ERα was less phosphorylated at Ser-118 after inhibition of the proteasome.

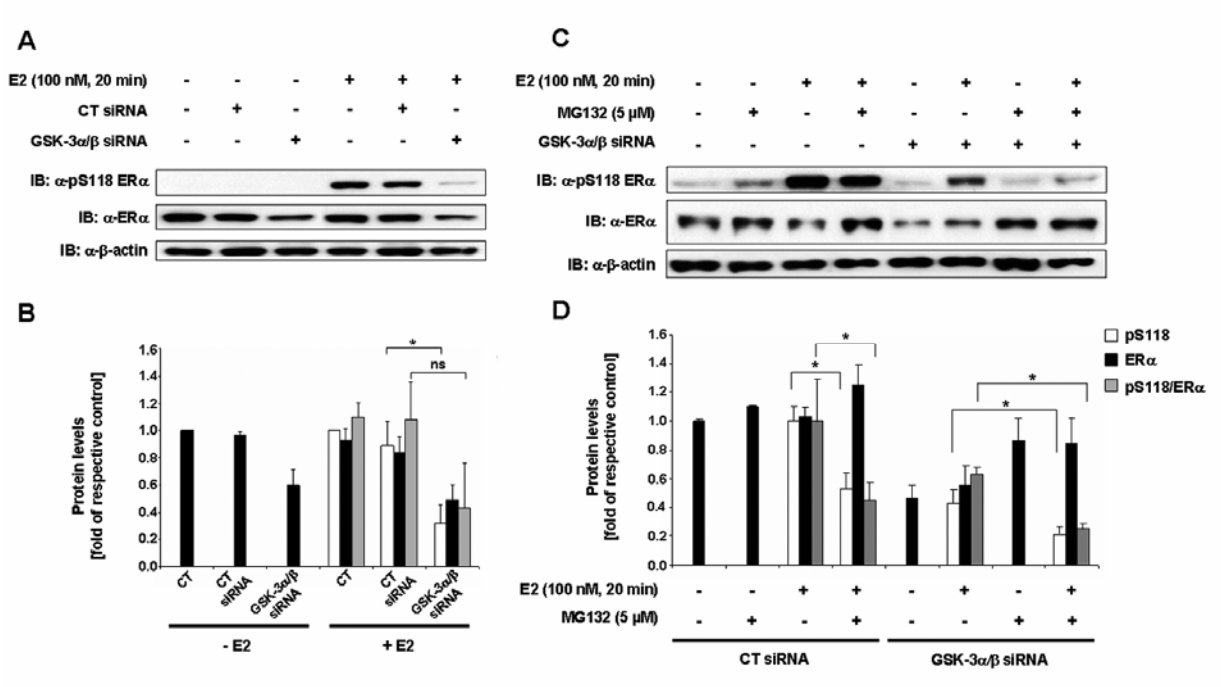


**Figure IV - 13: GSK-3 phosphorylates ERα at Ser-118 in MCF-7 cells.** ***Panel A.*** Cells were left untransfected (CT) or were transfected either with GL3 control siRNA (CT siRNA) or with siRNA targeting GSK-3α/β (GSK-3α/β siRNA) and treated or not with 100 nM E2 for 20 min. Immunoblots (IB) show reduction of E2-induced ERα phosphorylation at Ser-118 upon GSK-3 silencing. ***Panel B.*** Quantitative analysis of pS118 and ERα protein signals given as fold of control. Data from three independent experiments including that shown in panel *A* revealed reduction of Ser-118 phosphorylation, of ERα protein, as well as of pS118/ERα ratio (*, $p < 0.05$ determined by *t*-test; ns, not significant). ***Panel C.*** Cells were transfected either with GL3 control siRNA or with siRNA targeting GSK-3α/β. Where indicated, cells were pretreated with 5 μM MG132 for 6 h and then treated or not with 100 nM E2 for 20 min. ***Panel D.*** Quantification of data shown in panel *C* and of two additional experiments carried out under the same conditions (*, $p < 0.05$ determined by *t*-test). E2-induced and GSK-3α/β silencing-related downregulation of ERα was rescued by MG132; but reduction of E2-induced Ser-118 phosphorylation in cells transfected with GSK-3α/β siRNA was not rescued.

Previous work from our group showed that GSK-3 and ERα are detected in both the cytoplasm and the nucleus of MCF-7 cells (Medunjanin *et al.*, 2005; De Servi *et al.*, 2005). **Fig. IV – 1**, **Fig. IV - 3** and **Fig. IV – 4** shown in this thesis confirm the cytoplasmic and nuclear localization of GSK-3 and ERα in serum-starved cells. We now studied the effect of GSK-3 silencing on intracellular localization of ERα. **Fig. IV - 14A** shows that transfection of control siRNA did not interfere with E2-induced nuclear translocation of ERα. In GSK-3 silenced cells the overall ERα fluorescence signal was markedly weaker whether the cells were treated with E2 or not, which agrees with results from western blotting. Moreover,

GSK-3 silencing did not seem to affect E2-induced nuclear translocation of ERα. Cell fractionation studies (**Fig. IV - 14B)** showed similar GSK-3α/β silencing in both compartments. In agreement with the results shown in **Fig. IV - 14A** E2-induced ERα nuclear translocation was still occurring in GSK-3 silenced cells. Furthermore, **Fig. IV - 14B** shows that GSK-3 silencing resulted in reduced E2-induced ERα phosphorylation at Ser-118 in the nucleus. Taken together, these data suggest a direct role of nuclear GSK-3 regarding ERα phosphorylation at Ser-118 in the nucleus.

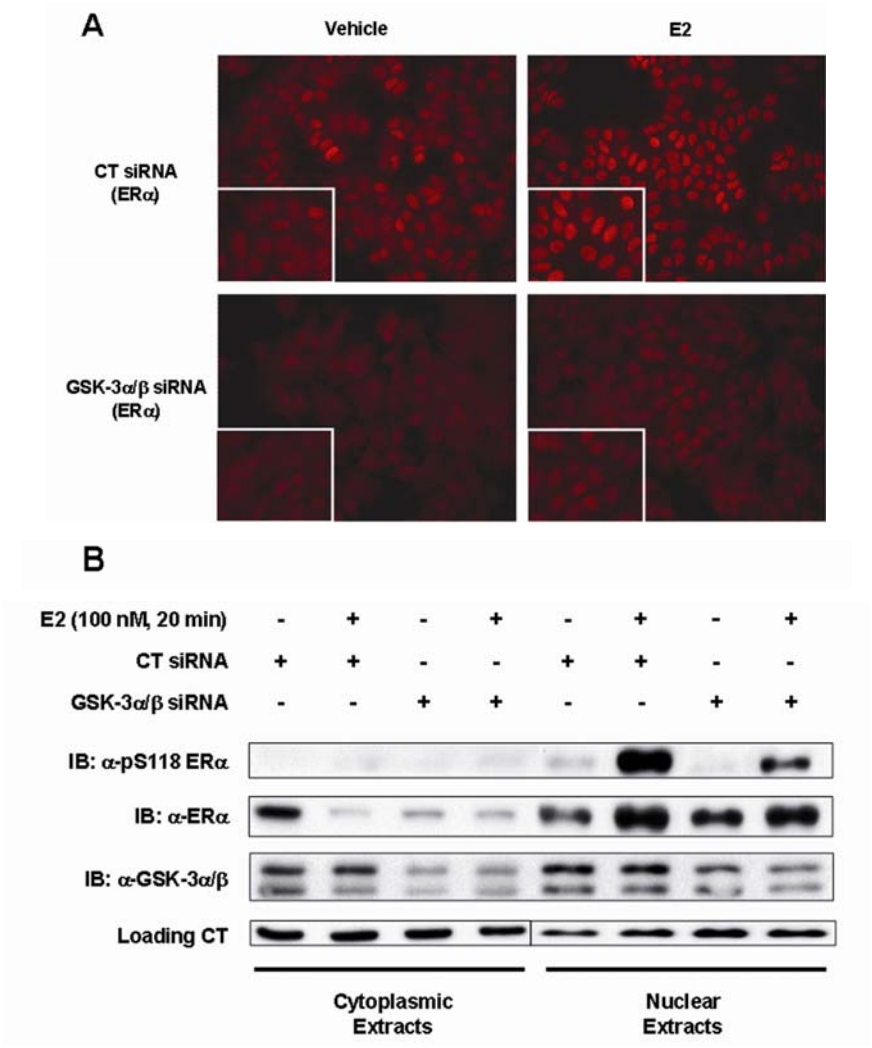


**Figure IV - 14: GSK-3α/β silencing did not alter E2-induced nuclear translocation of ERα.** Cells were transfected either with GL3 control siRNA or with siRNA targeting GSK-3α/β and treated or not with 100 nM E2 for 20 min. ***Panel A.*** Immunofluorescence staining of ERα protein (red) shows that cytoplasmic and nuclear ERα levels were reduced after GSK-3α/β silencing. E2-induced ERα translocation from the cytoplasm to the nucleus of MCF-7 cells was still occurring after GSK-3α/β silencing. The same settings were used for all fluorescence micrographs. ***Panel B.*** Cellular fractionation was performed and immunoblots (IB) show silencing of GSK-3α/β in both cytoplasmic and nuclear compartments upon RNAi. After GSK-3α/β silencing, E2-induced ERα phosphorylation at Ser-118 in the nucleus was decreased. β-actin and replication protein A / p34 were used as loading controls for cytoplasmic and nuclear extracts, respectively.

### IV - 4.6) Decrease of ERα activity due to GSK-3 silencing is not rescued by inhibition of the proteasome

Next, functional assays were performed to assess the role of GSK-3 in modulating ERα activity. First, MELN cells carrying an ERE-luciferase reporter plasmid were transfected with control siRNA or GSK-3α/β siRNA and treated with 10 nM E2 for different time periods before luciferase activity was measured. In control siRNA-transfected cells, luciferase activity was increased up to 7-fold after E2 treatment for 3 h, 6 h and 8 h and declined thereafter (**Fig. IV - 15A**). As expected, ERα was downregulated over time upon E2 treatment. In presence of GSK-3α/β siRNA, the basal luciferase activity (without E2 treatment) was significantly lower compared to the control at any time point analyzed. Moreover, E2-induced luciferase activity was significantly reduced by about 40 % in comparison with the respective controls when cells were treated with E2 from 3 h to 12 h. At 24 h, the luciferase activity after GSK-3 silencing was similar to the one obtained with control siRNA. Detection of ERα protein in the same samples used for luciferase reporter assay revealed reduction of ERα protein levels in cells transfected with GSK-3α/β siRNA compared to control siRNA-transfected cells. Further reduction of ERα protein content was observed by E2 treatment after GSK-3α/β silencing.

Next, we examined the effects of ERα rescue by proteasome inhibition on luciferase activity (**Fig. IV - 15B-C**). In agreement with results shown in **Fig. IV - 15A**, treatment of cells with 10 nM E2 for 6 h resulted in E2-induced luciferase activity, which was significantly inhibited in the presence of GSK-3α/β siRNA (**Fig. IV - 15B**). In control siRNA-transfected cells, addition of MG132 slightly increased basal luciferase activity but did not alter E2-induced luciferase activity. After GSK-3α/β silencing, the reduced E2-triggered luciferase activity was not rescued by MG132 treatment. However, ERα protein content in these cells was restored by MG132 treatment (**Fig. IV - 15C**). These findings show that GSK-3α/β is required for full ERα activation.

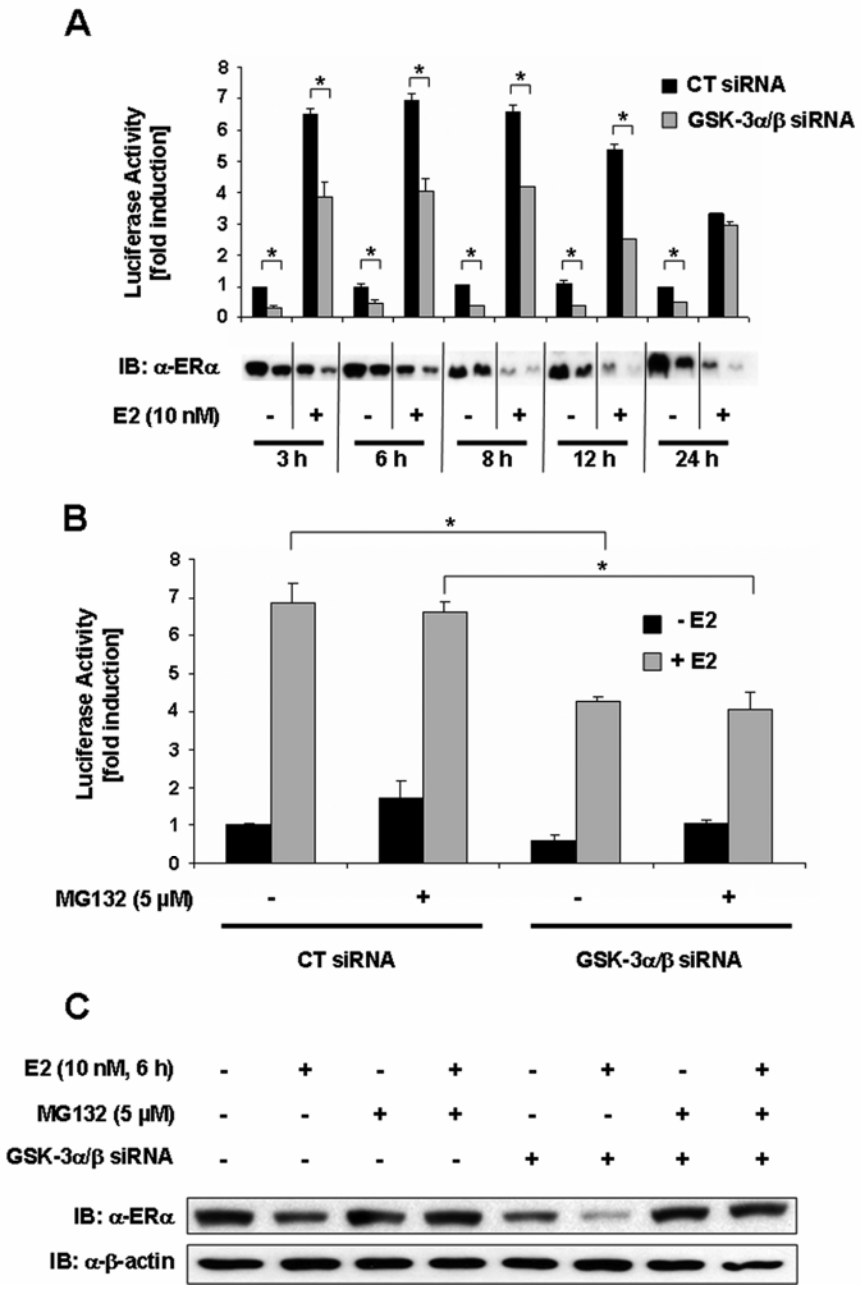


**Figure IV - 15: ERα transcriptional activity is decreased upon GSK-3α/β silencing.** ***Panel A.*** MELN cells transfected either with GL3 control siRNA (CT siRNA) or with siRNA targeting GSK-3α/β (GSK-3α/β siRNA) were treated with 10 nM E2 for 3, 6, 8, 12 or 24 h and ERE-dependent luciferase expression was measured in cell lysates. Relative luciferase activity given as fold of control was evaluated from three independent experiments (histogram). Immunoblot from the same lysates shows E2-induced and GSK-3α/β silencing-related downregulation of ERα (western blot). ***Panel B.*** Cells were transfected as above, pretreated with 5 µM MG132 for 1 h and treated or not with 10 nM E2 for 6 h. Relative luciferase activity was evaluated from three independent experiments (*, $p < 0.05$ determined by *t*-test). GSK-3α/β silencing-related reduction of E2-induced luciferase activity was not rescued by the addition of MG132. ***Panel C.*** Immunoblot (IB) showing that ERα protein in samples analyzed in panel B was restored by MG132 treatment.

### IV - 4.7) GSK-3α/β silencing decreases E2-induced expression of endogenous ERα target genes

Furthermore, we studied the effect of GSK-3 silencing on endogenous estrogen responsive genes. Quantitative real-time PCR was performed using primers specific for pS2 and progesterone receptor (PR), which are both well-known estrogen responsive genes (Klinge, 2001). After GSK-3α/β silencing, E2-induced expression of these genes was significantly decreased at 3 h, 6 h and 24 h of E2-treatment (**Fig. IV - 16A-B**). These results, in agreement with the luciferase assay performed in MELN cells, confirm the involvement of GSK-3α/β in the regulation of ERα target genes expression and demonstrate the necessity of GSK-3α/β for full ligand-dependent activity of ERα.

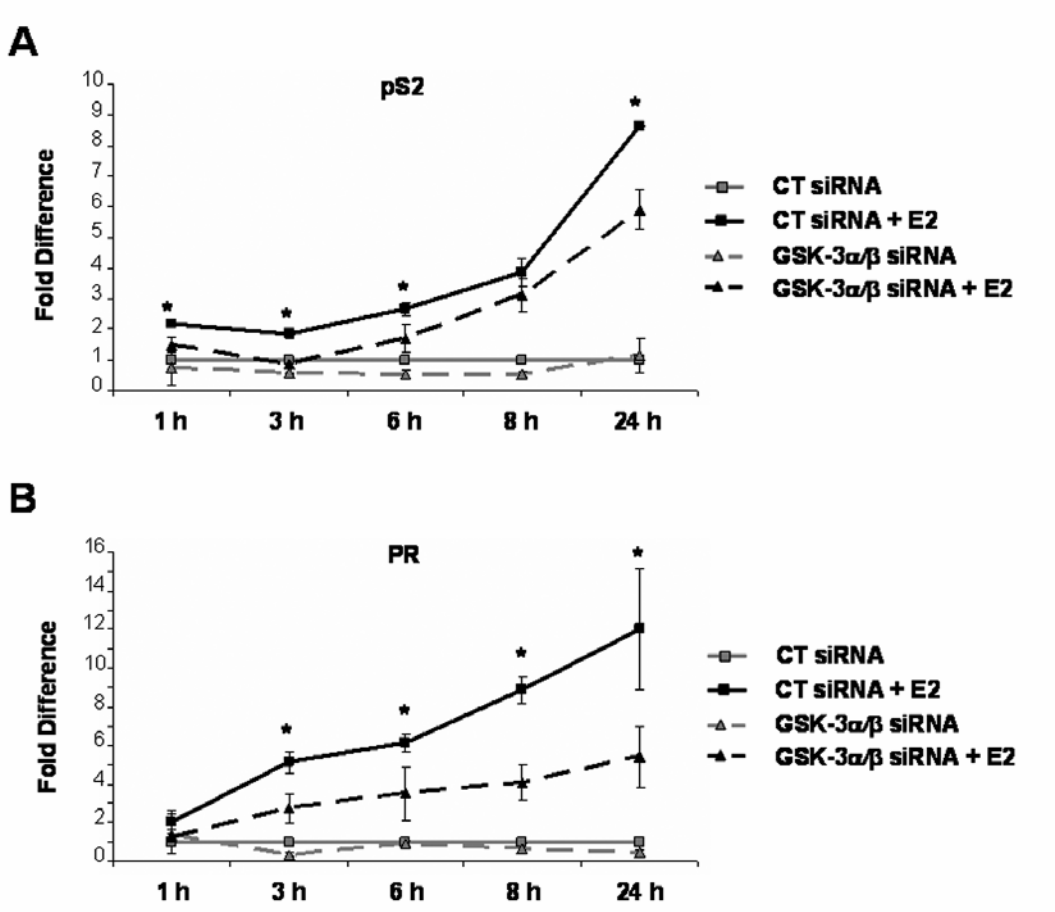


**Figure IV - 16: E2-induced expression of ERα target genes (pS2 and progesterone receptor) was downregulated upon GSK-3α/β silencing.** MCF-7 cells were transfected either with GL3 control siRNA (CT siRNA) or with siRNA targeting GSK-3α/β (GSK-3α/β siRNA) and then were treated or not with 10 nM E2 for 1 h, 3 h, 6 h, 8 h and 24 h. Total RNA was extracted and used to synthesize cDNA by reverse transcription. Quantitative real-time PCR of the cDNA using pS2 primers (***Panel A***) and progesterone receptor (PR) primers (***Panel B***) showed that E2-induced mRNA expression of these ERα target genes was decreased upon GSK-3α/β silencing. β-actin was used as an internal control. Three independent experiments were analyzed and error bars represent the standard error of the mean (*, $p < 0.05$ determined by *t*-test).

### IV - 4.8) Silencing of either GSK-3α or GSK-3β isoform results in ERα downregulation and reduced transcriptional activation

Since most reports in the literature claim that the GSK-3β isoform is of greater importance for biological functions in comparison with GSK-3α (Hoeflich *et al*., 2000; Grimes and Jope, 2001; Jope and Johnson, 2004), we were interested in the role of the two individual GSK-3 isoforms regarding ERα signalling. Indeed, a recent publication (Doble et al., 2007) highlights the importance of considering the contributions of both homologs when studying GSK-3 functions in mammalian systems. Therefore, the impact of individual GSK-3 isoforms on ERα protein level and activity were assessed using siRNA specifically targeting either GSK-3α or GSK-3β (**Fig. IV - 17A-C**). **Fig. IV - 17B** demonstrates the selective targeting of GSK-3α and GSK-3β by the respective siRNA. Selective silencing of GSK-3α resulted in ERα downregulation in the absence of E2 (**Fig. IV - 17A and 17B**) and in decrease of ERα phosphorylation at Ser-118 induced after 20 min E2 treatment (**Fig. IV - 17A**). After 6 h E2 treatment, selective silencing of GSK-3α led to a more pronounced E2-induced ERα downregulation (**Fig. IV - 17B**) and to a decrease of E2-induced luciferase activity (**Fig. IV - 17C**). The effects caused by GSK-3α silencing were similar to those described for GSK-3α/β silencing. Furthermore, selective silencing of GSK-3β had similar effects on ERα protein level, Ser-118 phosphorylation and ERα activity like GSK-3α or GSK-3α/β silencing (**Fig. IV - 17A-C**). Surprisingly, specific targeting of the GSK-3β isoform resulted also in partial silencing of GSK-3α. The lack of specificity of siRNA targeting GSK-3β is so far unclear. The results shown in **Fig. IV - 17** were obtained with the validated siRNA targeting GSK-3β from Ambion. Furthermore, similar results (**data not shown**) were obtained using the GSK-3β specific siRNA sequence described in (Phiel *et al.*, 2003).

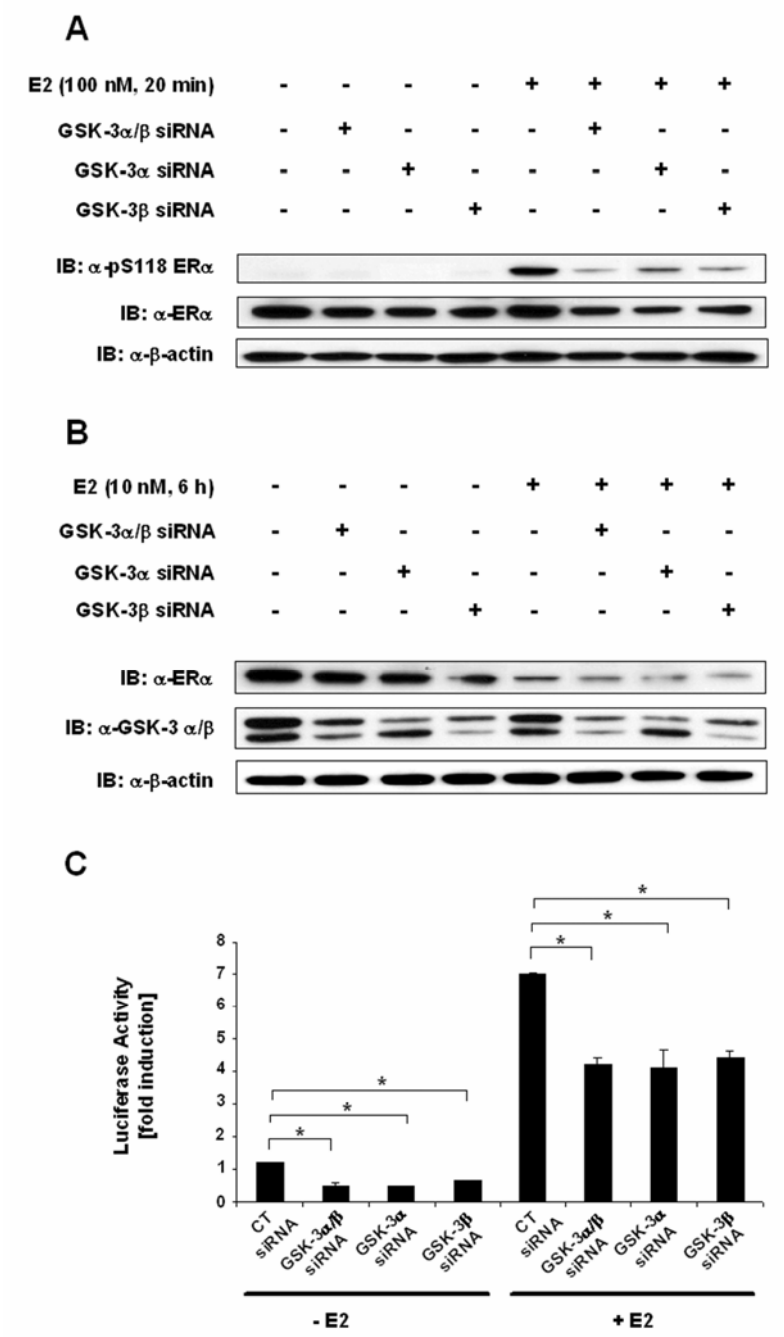


**Figure IV - 17: ERα level and activity are decreased upon silencing GSK-3α or GSK-3β individually.** Cells were transfected with 50 nM GL3 control siRNA (CT siRNA) or with 50 nM siRNA targeting either GSK-3α/β (GSK-3α/β siRNA), or GSK-3α (GSK-3α siRNA), or GSK-3β (GSK-3β siRNA). ***Panel A.*** Immunoblots (IB) showing ERα levels and Ser-118 phosphorylation in MCF-7 cells treated or not with 100 nM E2 for 20 min. ***Panel B.*** Immunoblots showing GSK-3α, GSK-3β and ERα levels in MCF-7 cells treated with 10 nM E2 for 6 h. ***Panel C.*** Relative luciferase activity given as fold of control was evaluated from three independent experiments in MELN cells treated with 10 nM E2 for 6 h and error bars represent the standard error of the mean (*, $p < 0.05$ determined by *t*-test).

We performed an additional experiment in which GSK-3α/β silencing was compared with the combined silencing of GSK-3α and GSK-3β individual isoforms (**Fig. IV - 18**). Both

approaches yielded similar results regarding GSK-3α/β downregulation (about 60 % at 50 nM final siRNA concentrations), GSK-3α/β silencing related ERα downregulation as well as GSK-3α/β silencing related decrease of E2-induced ERα activity. To summarize, similar results were obtained using different siRNA sequences specific for GSK-3α/β or the individual isoforms regarding ERα signalling.

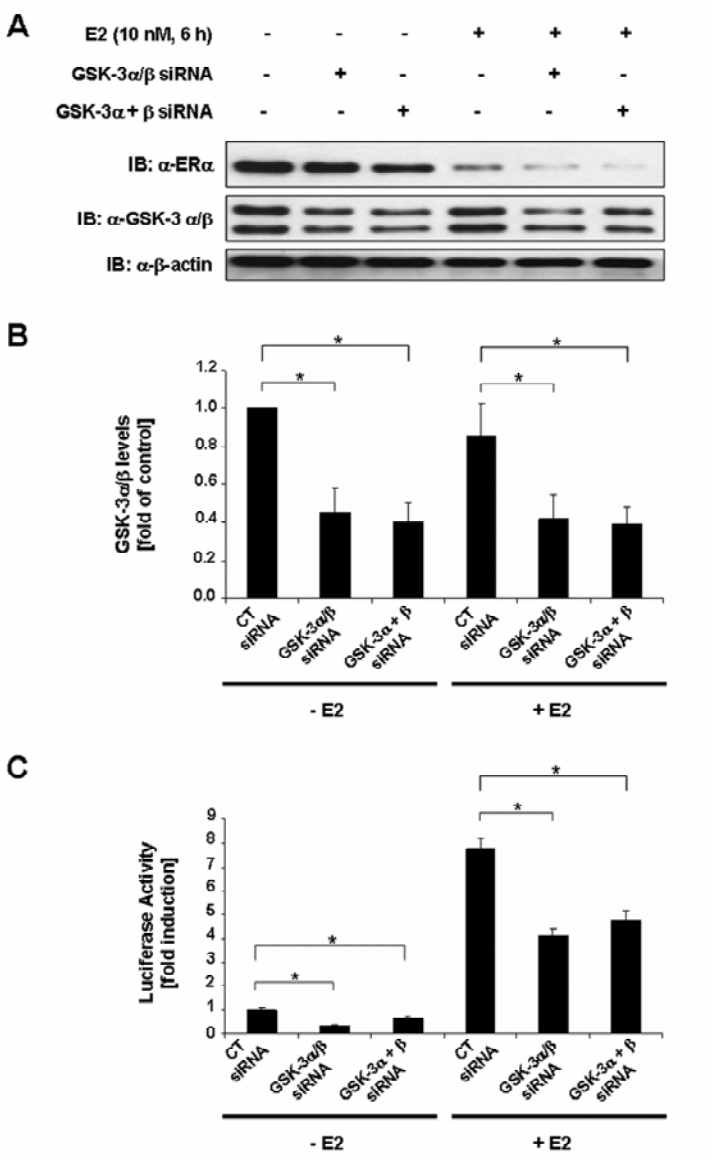


**Figure IV - 18: siRNA targeting GSK-3α/β or mix of siRNA targeting GSK-3α and siRNA targeting GSK-3β decreased ERα level and activity.** MELN cells were transfected with 50 nM GL3 control siRNA (CT siRNA) or with 50 nM siRNA targeting GSK-3α/β (GSK-3α/β siRNA), or with a mix of 25 nM siRNA targeting GSK-3α and 25 nM siRNA targeting GSK-3β (GSK-3α + β siRNA). Then, the cells were treated or not with 10 nM E2 for 6 h. ***Panel A.*** Immunoblots (IB) showing ERα levels after GSK-3α/β silencing. β-actin was used as loading control. ***Panel B.*** Quantitative analysis of GSK-3α/β immunoblots corrected for potential loading variations using β-actin. Three independent experiments were analyzed (*, $p < 0.05$ determined by *t*-test). ***Panel C.*** Relative luciferase activity given as fold of control was evaluated from three independent experiments in MELN cells (*, $p < 0.05$ determined by *t*-test).

### IV - 4.9) GSK-3 silencing causes increase of β-catenin protein content and enhances proteasomal degradation of cyclin D1

GSK-3β plays a crucial role in the regulation of the Wnt signalling pathway and induces β-catenin proteasomal degradation (Cohen and Goedert, 2004). Moreover, GSK-3β was shown to trigger proteolysis of cyclin D1 (Diehl *et al*., 1998). Therefore, we investigated the effects of GSK-3α/β silencing on these specific substrates of GSK-3 kinase.

In cells transfected with GSK-3α/β siRNA, β-catenin phosphorylation at Ser-33/37/Thr-41 was decreased and β-catenin protein content was increased (**Fig. IV - 19A**). In agreement with the findings from Cohen and Goedert (2004), these data showed that GSK-3 phosphorylates β-catenin at Ser-33/37/Thr-41 thus triggering the proteasomal degradation of this transcription factor. Additionally, these results confirmed the efficiency of the GSK-3 silencing.

Interestingly, after transfection with siRNA targeting GSK-3α/β, cyclin D1 phosphorylation at Thr-286 was decreased and down-regulation of cyclin D1 protein content was observed (**Fig. IV - 19A**). Additionally, cyclin D1 was rescued by inhibition of the proteasome with MG132 (**Fig. IV - 19B**). Taken together, these results suggest that GSK-3 phosphorylates cyclin D1 at Thr-286 and that GSK-3 may prevent proteolysis of cyclin D1. These results are at variance with previous studies (Diehl *et al*., 1998; Alt *et al*., 2000) which reported that GSK-3 induces Thr-286 phosphorylation of cyclin D1 and thereby triggers its proteasomal degradation. In our experiments, breast cancer cell lines were used, whereas the other papers mentioned used NIH-3T3 mouse fibroblast and Hela human cervix carcinoma cell lines. This may explain the differences of results.

To summarize, GSK-3 interacts with, phosphorylates and induces proteasomal degradation of β-catenin. Moreover, similar to the data we obtained regarding ERα/GSK-3 interaction and stabilization, GSK-3 may interact with and protect cyclin D1 from proteasomal degradation. Additionally, the phosphorylation of cyclin D1 at Thr-286 by GSK-3 may play a role in the protection of cyclin D1 from proteolysis. Hypothetically, this phosphorylation may reinforce the putative interaction between GSK-3 and cyclin D1.

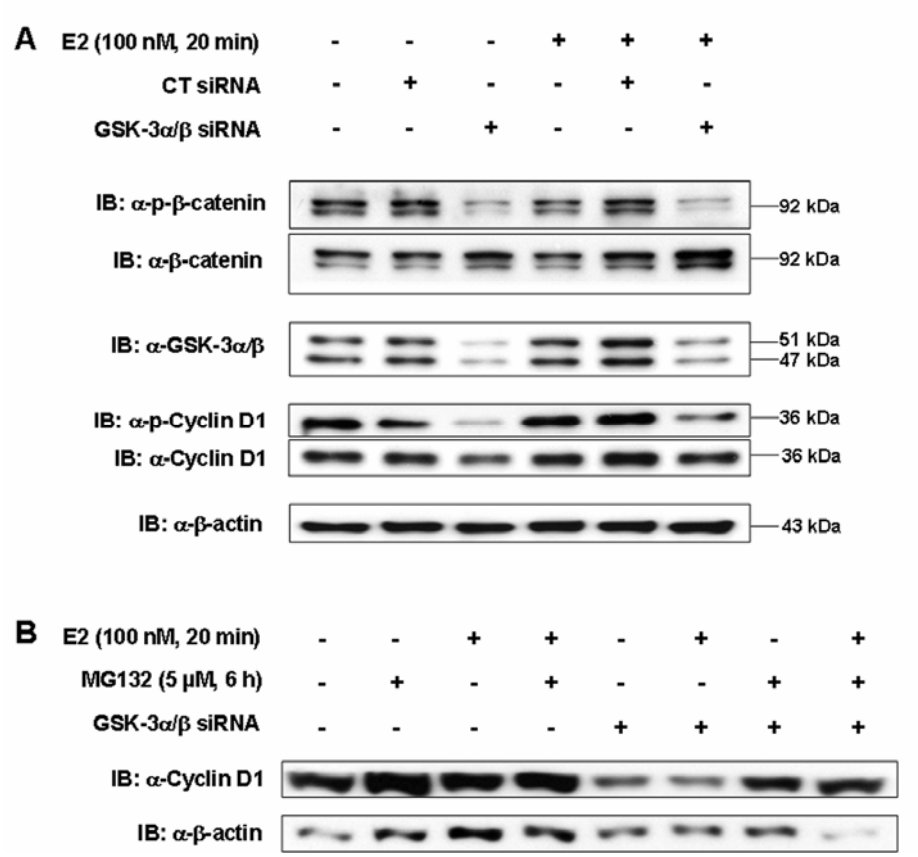


**Figure IV - 19: GSK-3 silencing-related increase of β-catenin protein content and GSK-3 silencing-related increase of cyclin D1 proteasomal degradation in MCF-7 cells.** ***Panel A.*** Cells were left untransfected or were transfected either with GL3 control siRNA (CT siRNA) or with siRNA targeting GSK-3α/β (GSK-3α/β siRNA) for 66 h, and were then treated or not with 100 nM E2 for 20 min. After GSK-3 silencing, in absence or in presence of E2, immunoblots (IB) show reduction of β-catenin phosphorylation at Ser-33/37 and Thr-41 and consequent increase of β-catenin protein content, whereas cyclin D1 phosphorylation at Thr-286 and protein levels were both decreased. β-actin was used as loading control. ***Panel B.*** Cells were transfected either with GL3 control siRNA or with siRNA targeting GSK-3α/β. Where indicated, cells were pretreated with 5 μM MG132 for 6 h and then treated or not with 100 nM E2 for 20 min. Immunoblot shows rescue by proteasomal inhibitor MG132 of GSK-3α/β silencing-related downregulation of cyclin D1. E2 treatment does not seem to alter the downregulation and the rescue of cyclin D1 previously described. β-actin was used as loading control.

## IV - 5) GSK-3β WT, CA, R96A and R96K enhanced E2-induced ERE-dependent luciferase activity of MELN cells

To determine the relevant amino acid residues or domains of GSK-3 involved in modulation of ERα activity, MELN cells were transiently transfected with expression constructs carrying different GSK-3β mutants. The GSK-3β WT (Wild Type) form was used as a control for overexpression of the kinase. The GSK-3β CA (constitutively active) form was obtained by mutation of the Ser-9 to Ala- (S9A). The mutation of Arg-96 to Ala (R96A) or Lys (R96K) abolished GSK-3β activity toward primed substrates, but these mutations did

not alter GSK-3β activity toward non-primed substrates (Frame *et al*., 2001). To check the expression levels of the flag-tagged GSK-3 constructs, western blot analysis was performed with the cell lysates used for luciferase activity measurement later on (**Fig. IV - 20A**). All the constructs had a similar flag expression pattern and their expression was not altered by 48 h of E2 treatment. In the same lysates, GSK-3 protein level was also detected by immunoblot analysis. GSK-3β protein level was increased after transfection of the cells with WT, CA, R96A or R96K GSK-3β constructs in comparison with cells transfected with an empty vector (Mock). Interestingly, the increase of the GSK-3β protein level was always accompanied by an increase of protein level for the GSK-3α isoform. Additionally, our results suggest for each GSK-3 construct studied, a slight increase of protein levels for both GSK-3 isoforms in E2 treated cells in comparison with untreated cells.

Furthermore, we tested the effect of these GSK-3β constructs on ERE-dependent luciferase activity in MELN cells. In these experiments, the constructs were co-transfected with β-galactosidase which was used as an internal control for transfection efficiency while quantifying the luciferase activity (**Fig. IV - 20B**). In absence of E2, a slight increase of basal luciferase activity was observed after overexpression of GSK-3β WT, R96A and R96K constructs. The CA GSK-3β construct did not seem to alter the basal ERE-dependent luciferase expression. In cells treated for 48h with E2, overexpression of GSK-3β WT, R96A and R96K resulted in significant increase of luciferase activity (about 3-fold) in comparison with the Mock control. GSK-3β CA showed only about 1,5-fold increase of luciferase activity compared to the Mock control).

All together, these data suggested that GSK-3β overexpression increased ERα activity upon E2 treatment and that ERα may be an un-primed substrate of GSK-3β. Moreover, phosphorylation of GSK-3β at Ser-9 seemed to be important for full E2-induced ERα activity. Additionally, effects of GSK-3 constructs on E2-induced ERα nuclear translocation and Ser-118 phosphorylation were studied, but no clear conclusions were possible (**data not shown**). Furthermore, the endogenous GSK-3α and GSK-3β were still strongly effective in these experiments and did not allow a specific study of each construct.

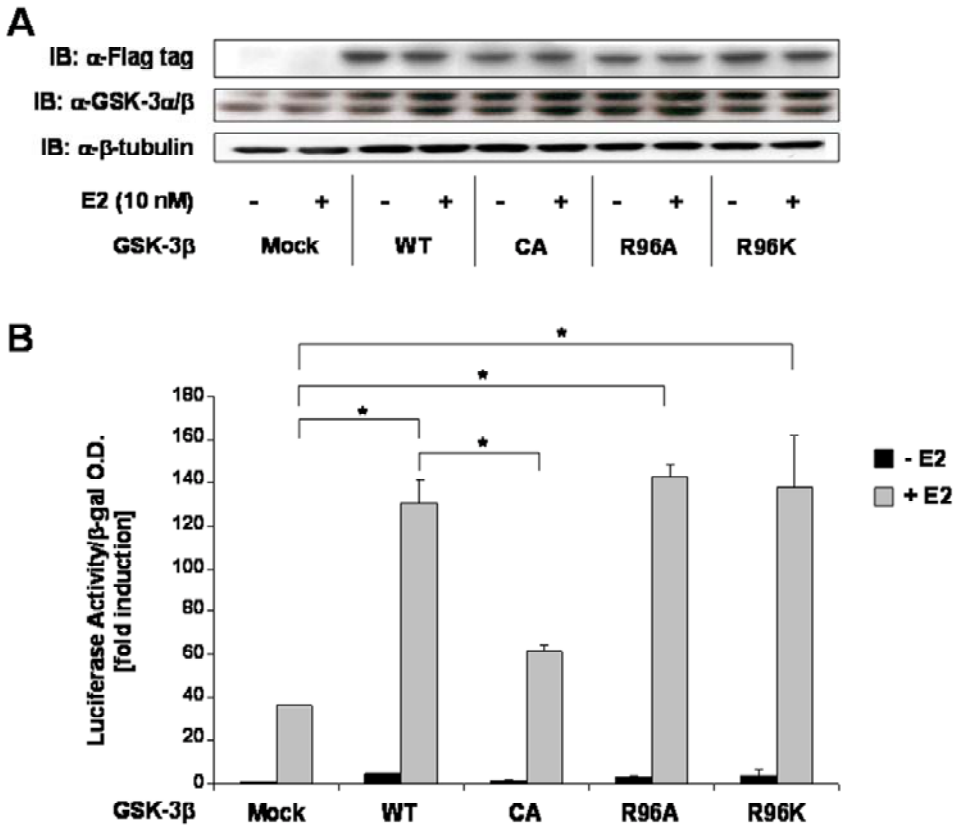


**Figure IV - 20: GSK-3β WT, CA, R96A and R96K enhanced E2-induced ERE-dependent luciferase activity of MELN cells.** MELN cells were transiently co-transfected with empty vector (Mock), expression vectors carrying wild-type (WT) or mutant GSK-3β such as constitutively active (CA), R96A or R96K GSK-3β constructs and a β-galactosidase expression vector. Then, these cells were incubated for 48 h with E2 (**10 nM**). ***Panel A.*** Immunoblots (IB) of cell lysates show expression of the different flag-tagged GSK-3β constructs. In comparison with Mock transfected cells, increased expression of GSK-3 protein level was observed in WT, CA, R96A and R96K GSK-3β transfected cells. β-tubulin was used as loading control. ***Panel B.*** In the same lysates used previously for determination of GSK-3 overexpression, ERE-dependent gene expression was quantified by measuring luciferase activity. Data are expressed as fold of the luciferase activity measured in untreated Mock-transfected cells. These data were normalised by the measurement of the β-galactosidase activity of the cells (β-gal O.D.). Error bars represent standard deviation from three independent experiments in MELN cells (*, $p < 0.05$ determined by *t*-test).

## IV - 6) Silencing of the endogenous GSK-3β in MCF-7 cells using miR RNAi expression vectors and rescue of the kinase and ERα with xenopus GSK-3β constructs

As described above, the effects of overexpressed GSK-3β mutants on ERE-controlled luciferase activity were difficult to interpret since endogenous GSK-3β was still present and interfered with the mutants. The investigation of the effect of GSK-3β mutants on ERα stability and function required the elimination of endogenous GSK-3β. Therefore, the aim was to silence the endogenous human GSK-3 kinase using miR expression constructs and to

overexpress specifically the GSK-3β mutants of interest. In order to avoid targeting and degradation of the transfected mutants by the GSK-3β-targeting miR sequences, GSK-3β constructs with sequences from xenopus were used for transfection.

### IV - 6.1) Evaluation of the efficiency of miR RNAi expression vectors regarding GSK-3β silencing to select the most appropriate candidate

Three promising miR sequences targeting human GSK-3β were selected using the criteria advised by the Wadsworth Bioinformatics Centre website interface. Moreover, the miR candidates chosen should avoid targeting of the human GSK-3α isoform and of the xenopus GSK-3α and GSK-3β isoforms. The specificity of the candidate sequences for human GSK-3β was checked using the HUSAR and NCBI interfaces. The 741 miR, 599 miR and 1839 miR were selected and cloned into pcDNA6.2 vectors expressing the green fluorescence protein (GFP). After transfection of MCF-7 cells with the miR constructs, GSK-3β silencing was observed using immunofluorescence, quantitative PCR and western blot analyses. **Fig. IV - 21** shows a representative field of cells transfected with the different constructs and assessed with double fluorescence. The green fluorescence shows the GFP expression of the constructs in transfected cells and the red immunofluorescence evaluates the GSK-3β protein levels of the cells using an anti-GSK-3β specific antibody. Overlay of red and green immunofluorescence indicates the efficiency of GSK-3β silencing in transfected cells. Cells transfected with the control miR construct showed an orange colour overlay reporting a lack of GSK-3β silencing. For the candidate miRs, a green colour overlay demonstrated that GSK-3β expression was greatly reduced in cells expressing GFP due to the presence of the miR. The fluorescence overlay results suggested that the 599 miR was the best candidate to silence endogenous GSK-3β. The 1839 miR was also efficient and it seemed that the 741 miR was only weakly reducing GSK-3β protein level.

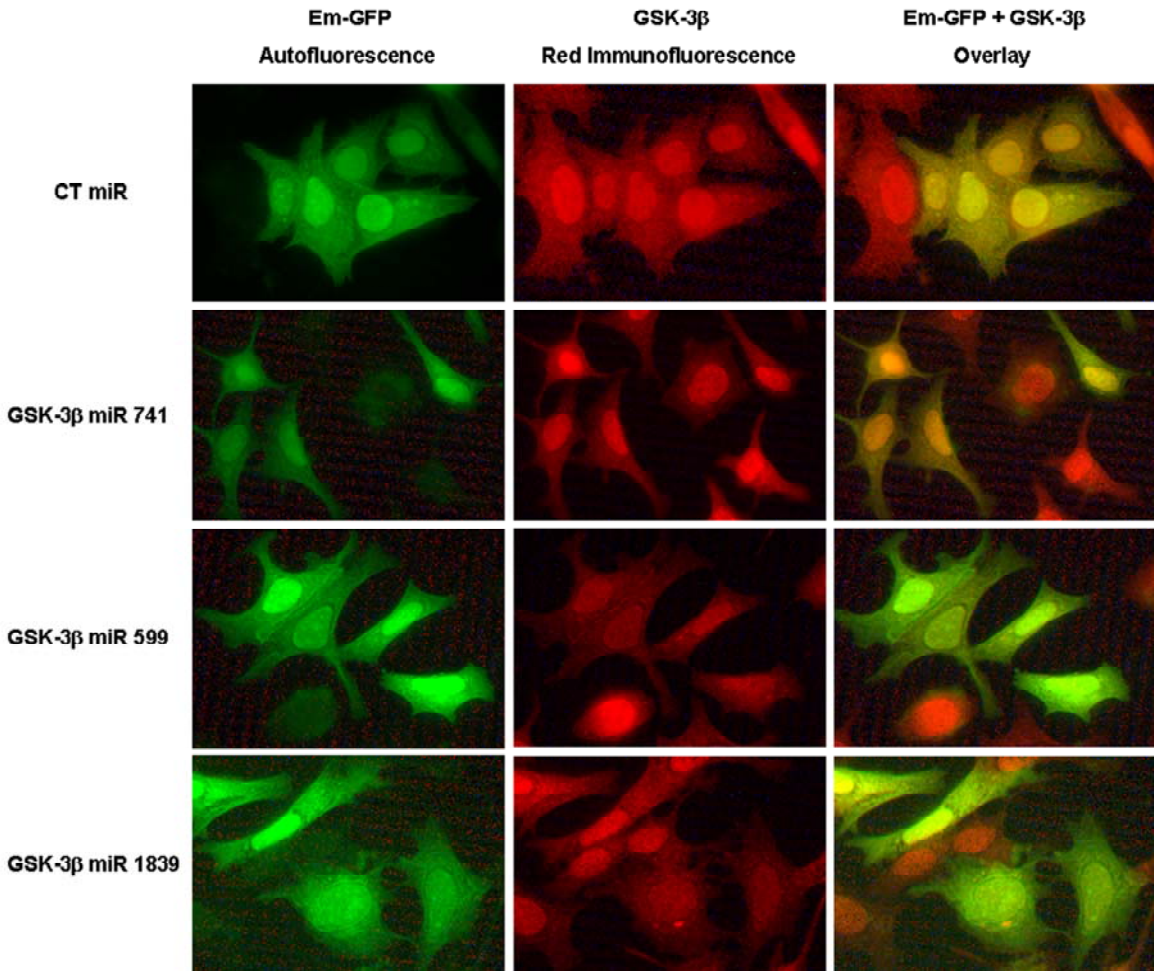


**Figure IV - 21: GSK-3β silencing using miR expression vectors.** MCF-7 cells were transfected either with miR expression vector targeting the mouse brain glucocorticoid receptor GPR54 and used as a negative control (CT miR) or with GSK-3β miR expression vectors (GSK-3β miR 741, GSK-3β miR 599 and GSK-3β miR 1839) targeting different sequences of the human GSK-3β gene. After 48 h transfection, cells were fixed and stained. The same field of cells is shown in each row to reveal EmGFP (auto-fluorescence), GSK-3β (immunofluorescence using rabbit GSK-3β primary antibody and Cy3 labelled anti-rabbit secondary antibody), and the overlay of EmGFP and GSK-3β fluorescences. Transfection efficiency reached about 50 % for each construct (evaluation of GFP tagged cells vs. the total number of cells per microscopic field). The orange overlay observed for the CT miR transfected cells suggests the presence of both miR expression vector and GSK-3β protein in these cells. This overlay colour was also observed for most of the GSK-3β miR 741 transfected cells. For the GSK-3β miR 599 and the GSK-3β miR 1839 expression vectors, the majority of the cells expressing GFP do not appear to have GSK-3β present, and cells stained red for GSK-3β do not appear to have any GFP expression. This demonstrates that cells expressing EmGFP miR 599 and EmGFP miR 1839 are greatly reduced in GSK-3β expression due to the presence of the miR.

The 599 miR was confirmed to be a highly potent candidate reducing GSK-3β mRNA expression quantified by real-time PCR (**Fig. IV - 22A**), and GSK-3β protein level analysed by western blotting (**Fig. IV - 22B**). The 1839 miR was also efficient in decreasing GSK-3β mRNA and protein levels and the 741 miR was confirmed to poorly silence GSK-3β.

Surprisingly, although care was taken when choosing the miR, reduced GSK-3α isoform protein levels (**Fig. IV - 22B**) were also observed each time when the GSK-3β isoform was successfully silenced. This agrees with data obtained by GSK-3β silencing using siRNA (see **Fig. IV - 17B**). Taken together, these data suggest that the GSK-3β isoform may be involved in the regulation of the GSK-3α isoform protein content. Additionally, these data reveal that the strongest silencing of GSK-3β was achieved using the 599 miR expression vector.

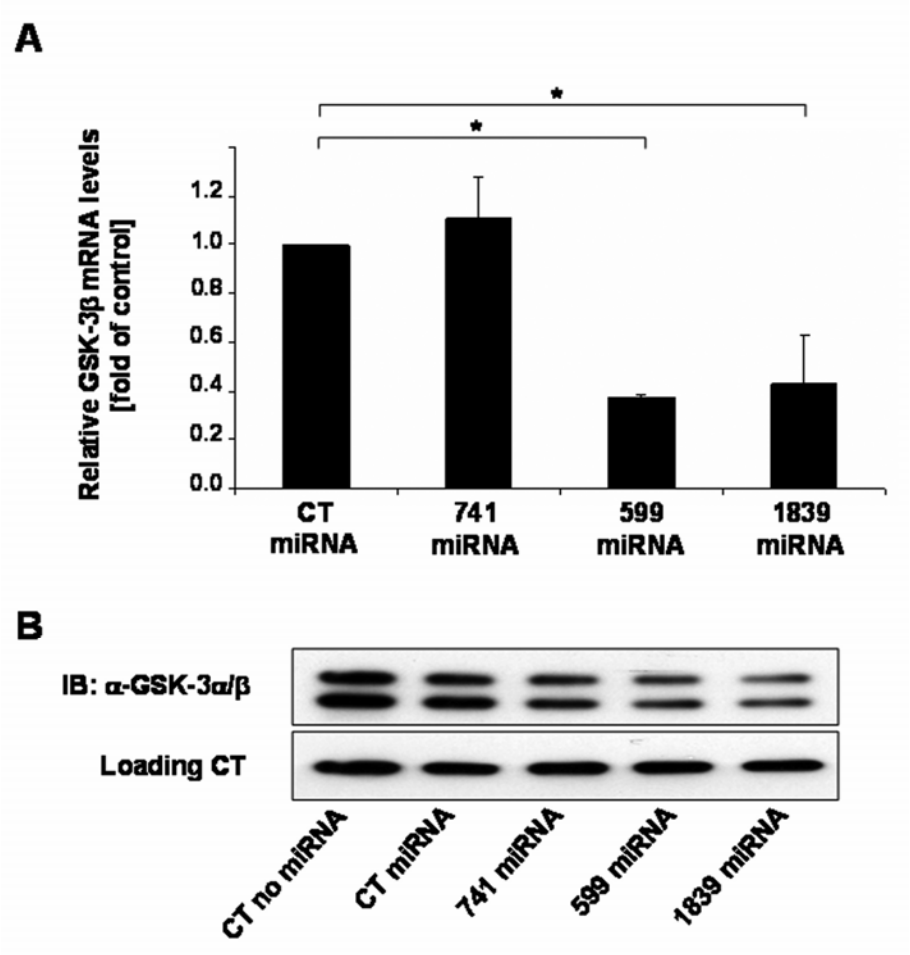


**Figure IV - 22: Silencing of GSK-3β was efficient with the 599 miR and the 1839 miR expression vectors.** ***Panel A.*** MCF-7 cells were transfected either with CT miRNA plasmid or with expression vectors targeting GSK-3β (741 miRNA, 599 miRNA and 1839 miRNA). 66 h post-transfection, total RNA was extracted and used to synthesize cDNA by reverse transcription. Quantitative real- time PCR using GSK-3β primers showed that miR-related decrease of GSK-3β mRNA expression was significantly achieved upon 599 miRNA and 1839 miRNA transfection. β-actin was used as an internal control. Three independent experiments were analyzed and error bars represent the standard error of the mean (*, $p < 0.05$ determined by *t*-test). ***Panel B.*** MCF-7 cells were left untransfected (CT no miRNA) or were transfected either with CT miRNA plasmid or with expression vectors targeting GSK-3β (741 miRNA, 599 miRNA and 1839 miRNA). Lysates were obtained after 66 h of transfection. Immunoblots (IB) showing GSK-3α/β protein levels confirmed the results obtained in *Panel A*. β-actin was used as loading control.

### IV - 6.2) Rescue of endogenous human GSK-3β silencing with xenopus GSK-3β constructs and effect on ERα protein level

The 599 miR expression vector was used to silence endogenous GSK-3β. Co-transfection of the MCF-7 cells with different constructs of xenopus GSK-3β aimed to rescue the loss of endogenous GSK-3β and to find the domain or amino acid residues of GSK-3β relevant for protecting ERα from proteasomal degradation. GSK-3β is highly conserved between the human and the xenopus species (92 % similarity according to Forde and Dale, 2007) suggesting that the xenopus GSK-3β wild type (WT) may have a similar role in comparison with the human isoform and may be suitable to replace the human GSK-3β (**Fig. IV - 23A**). Moreover, key amino acids involved in the regulation of GSK-3β kinase are conserved between the two species suggesting that xenopus GSK-3β mutants will have the same effects as the human mutants. If this proofs true, the specific effects of each mutant regarding ERα function could be strictly determined without interference of the endogenous GSK-3β. While the 599 miR was shown to specifically target the human GSK-3β cDNA sequence, numerous mismatches of 599 miR with the xenopus GSK-3β cDNA sequence were encountered (**Fig. IV - 23B**), suggesting a specific silencing of the human isoform. Therefore, interference of the endogenous GSK-3β with the overexpressed xenopus GSK-3β is anticipated.

Using anti-GSK-3α/β antibody recognizing the human as well as the xenopus GSK-3 epitopes, we observed a rescue of the 599 miR-related silencing of the human GSK-3β isoform by the xenopus GSK-3β (**Fig. IV - 23C**). This rescue was shown for the WT and the dominant negative (DN or K85M/K86I) xenopus mutants and this rescue was confirmed using an antibody recognizing the GSK-3β isoform alone. Importantly, rescue of ERα protein content was observed after the rescue of the GSK-3 protein. Silencing of GSK-3β decreased ERα protein level, and rescue of GSK-3β with WT or DN xenopus constructs resulted in increased ERα. Overall, these results suggest that GSK-3β stabilizes ERα and stabilization is independent of GSK-3 kinase activity. Interestingly, GSK-3α overexpression was also observed when GSK-3β isoform was rescued. Together with the findings on GSK-3α down-regulation after GSK-3β silencing, this observation suggests a regulation of GSK-3α expression level by GSK-3β. Additionally, a role of the overexpression of the endogenous GSK-3α isoform in the rescue of the ERα protein cannot be excluded.

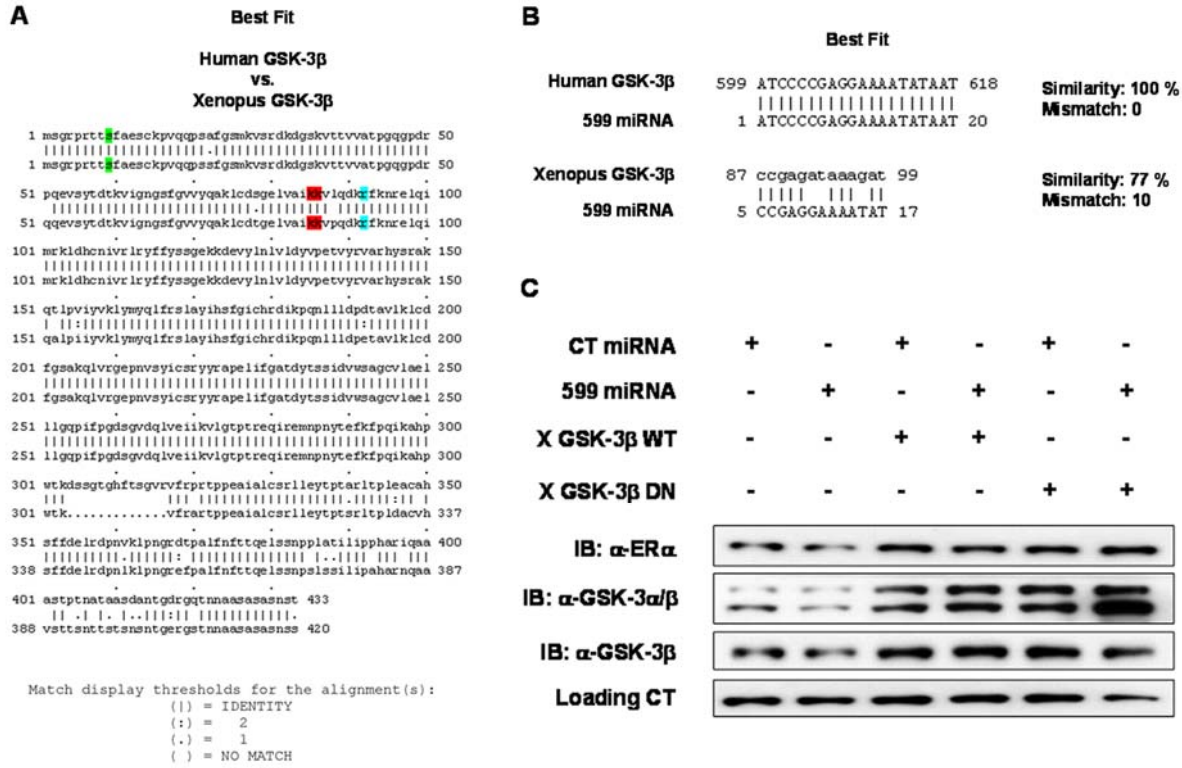


**Figure IV - 23: Xenopus GSK-3β rescue of 599 miR-silenced human GSK-3β and rescue of 599 miR-related ERα protein decrease.** ***Panel A.*** Using the HUSAR interface and the database from NCBI website, best fit was performed between amino acid sequences of the human GSK-3β (*upper line*) and the xenopus GSK-3β (*lower line*) showing that GSK-3β is highly conserved between these two species which suggests a similar role of the two proteins regarding GSK-3β substrates. For example, the Ser-9 residue (green), Lys-85 and Lys-86 residues (red) as well as Arg-96 (blue) residue are shown to be conserved. These residues are important for the phosphorylation and inactivation of GSK-3β, for kinase activity of the enzyme and for primed-phosphate substrate binding to its GSK-3β docking site, respectively. ***Panel B.*** Using the same websites as in *Panel A*, the DNA sequence related to the 599 miRNA construct was shown to be 100 % similar to the human GSK-3β and only 77 % similarity was achieved for the xenopus GSK-3β with 10 mismatches present. This proves that the 599 miRNA specifically targets the human GSK-3β. ***Panel C.*** MCF-7 cells were transfected with the control miR plasmid (CT miRNA) or with the 599 miR expression vector (599 miRNA), or MCF-7 cells were co-transfected with the CT miRNA or the 599 miR and either with the xenopus GSK-3β wild type construct (X GSK-3β WT) or with the 599 miR and xenopus GSK-3β dominant negative construct (X GSK-3β DN). Lysates were obtained after 66 h of plasmid transfection. Immunoblots (IB) showing 599 miR-related silencing of GSK-3β and rescue of this kinase by the xenopus constructs. Immunoblots also show that the 599 miR-related decrease of ERα was rescued by WT and DN xenopus GSK-3β expression. β-actin was used as loading control.

## IV - 7) GSK-3β protein expression pattern in human breast cancer

The ultimate aim of the project will be to prove that the GSK-3β/ERα signalling pathway depicted in immortalized cell culture lines may have some impact on breast tumorigenesis. Therefore, we first investigated the expression of GSK-3β protein in breast

cancer samples. Immunohistochemistry was performed on human breast tissue sections that contained tumors of different grades in order to determine the expression pattern of GSK-3β protein according to the tumor grade.

### IV - 7.1) From normal breast tissue to breast cancer

The histological evaluation and grading of patient's pre-cancerous and cancerous breast tissues is an important diagnostic performed by pathologists to orientate the patient in the most appropriate treatment strategy. After surgery, histological diagnosis represents a powerful tool to estimate complete removal of the tumor. Histopathological grading of the tumor contributes to determine the strategy of the patients' treatment. Grade 1 tumors represent cancer with slower cell growth, high differentiation status and better prognosis, whereas cancers with faster cell growth, low differentiation status, bad prognosis and high probability of recurrence are classified into grade 3.

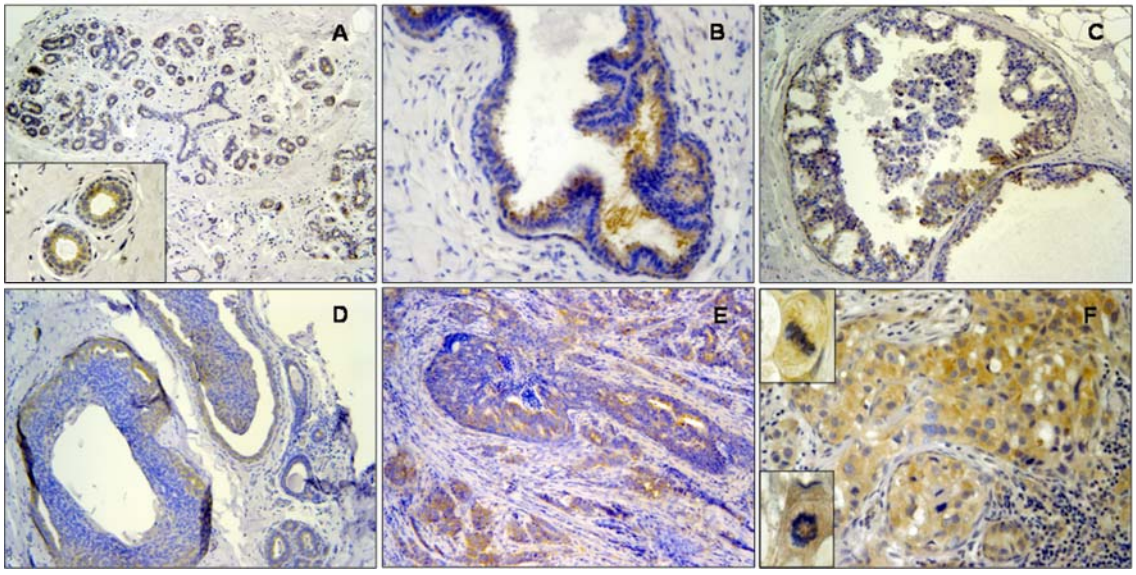


**Figure IV - 24: Expression of GSK-3β in normal human mammary tissues and breast cancers.** Immunohistochemical staining using GSK-3β antibody (yellow to brown colour developed by DAB peroxidase reaction) and hematoxylin counterstaining (blue colour) on formalin-fixed paraffin sections from breast cancer patients. Normal mammary tissue (***A***) showing a central duct surrounded by lobules consisting of alveoli (inset showing higher magnification). The outer layer of the alveolus is formed by myoepithelial cells while the inner layer is composed by luminal epithelial cells. Benign hyperplastic alterations, i.e. papilloma-like and apocrine cyst-like structures are shown in (***B***) and (***C***), respectively. These pre-cancerous lesions may evolve into cancer. Intraductal grade 1 tumor cell mass is shown in (***D***). (***E***) shows a grade 2 ductal invasive carcinoma. The structure of the duct is destroyed and tumor cells are invading the stromal tissue of the breast. A highly malignant grade 3 tumor (***F***) shows nuclear polymorphism and variation in nuclear size as well as mitotic figures. The inset in the *upper left corner* shows a metaphase and the inset in the *lower left corner* shows an aberrant mitotic figure with annular arrangement of chromosomes in metaphase. [Magnifications: *Panel A*: x 50 (inset: x 160); *Panel B*: x 160; *Panel C*: x 160; *Panel D*: x 100; *Panel E*: x 100; *Panel F*: x 200 (insets: x 400)].

Normal mammary tissue is shown in **Fig. IV - 24A**. We observe that the normal breast consists of ducts and lobules embedded into stromal tissue. Higher magnification of two lobular alveoli is shown in the inset. Most breast tumors arise from the ducts of the mammary glands. Hyperplasia may arise within the ducts (**Fig. IV - 24B-C**) and this pre-cancerous lesion may evolve into cancer. First, intraductal growth of tumors is observed. **Fig. IV - 24D** shows a grade 1 intraductal tumor. Then, cancer cells will cross beyond the ductal cell layers and the basement membrane to invade the breast stromal tissue (**Fig. IV - 24E**). Finally, highly malignant grade 3 tumors are observed (**Fig. IV - 24F**). A hallmark of grade 3 tumors is a strong aberration in the morphology and size of the nuclei as well as an increased number of mitotic figures (**Fig. IV - 24F**). Besides regular mitotic figures (**Fig. IV - 24F, upper left corner**), aberrant mitotic figures (**Fig. IV - 24F, lower left corner**) are observed.

In normal tissue, a weak and cytoplasmic-localised staining of GSK-3β protein was observed in both lobular and ductal epithelial cells. In pre-cancerous and cancerous lesions, various intensities were detected. To statistically evaluate GSK-3β protein content according to the tumor grade, scoring of the GSK-3β staining intensity was performed thereafter.

### IV - 7.2) Scoring of the GSK-3β immunohistochemical staining intensity

Immunohistochemical staining of GSK-3β protein was performed and the intensity of the staining was scored as (0) for negative/faint staining, (1) for very weak/weak staining and (2) for moderate/strong staining (**Fig. IV - 25**). Since GSK-3β protein was mainly detected in the cytoplasm of the tumor cells, the scoring was based on the cytoplasmic staining of GSK-3β. Only rarely and exclusively for the scoring intensity of (2), nuclear staining of tumor cells were observed. Primarily, five scores were considered, but thereafter, the very weak and weak as well as the moderate and strong staining intensities were regrouped together into scores 1 and 2, respectively. The aim was to increase the number of cases for each score.

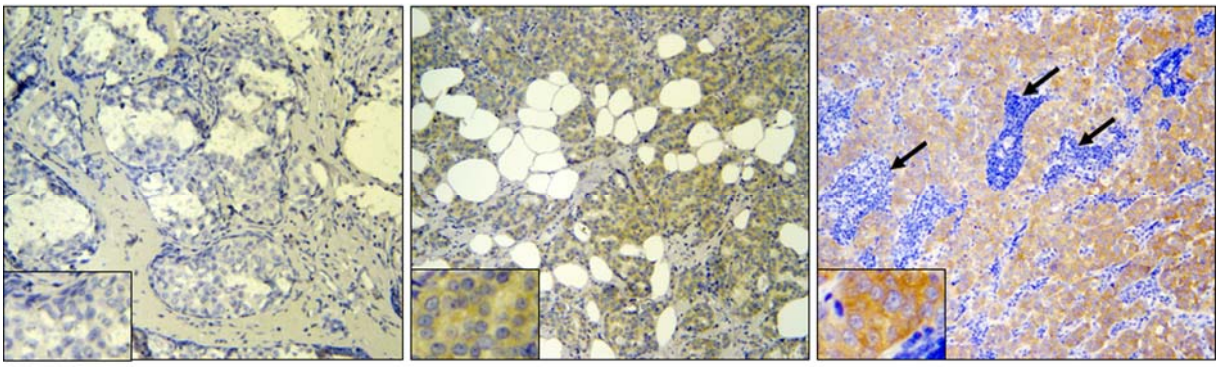


**Figure IV - 25: Immunohistochemical staining of GSK-3β and scoring of the staining intensity.** Using a monoclonal antibody, GSK-3β was detected in formalin-fixed paraffin sections from grade 1/2 and grade 3 breast cancer tissues and the intensity of the staining was scored as indicated for the representative fields shown (magnification x 100): negative to faint (score 0, ***left panel***), very weak to weak (score 1 or yellow colour, ***middle panel***) and moderate to strong (score 2 or orange/brown colour, ***right panel***). For each score, a representative field is shown at higher magnification (x 200). GSK-3β is mainly located to the cytoplasm. Inflammatory cells often observed in breast cancer appear negative (black arrows, ***right panel***).

Immunohistochemical staining of GSK-3β was evaluated semi-quantitatively according to the previously described scoring. 29 tumor samples from German patients (16 cases of grade 1/2 and 13 cases of grade 3) and 43 tumor samples from Chinese patients (23 cases of grade 1/2 and 20 cases of grade 3) were analyzed (**Fig. IV - 26**).

In the samples from German patients, among the grade 1/2 tumors, 4 cases had a GSK-3β scoring intensity of (0), 8 cases had a GSK-3β scoring intensity of (1) and 4 cases had a GSK-3β scoring intensity of (2). Among the grade 3 tumors, 2 cases had a GSK-3β scoring intensity of (0), 3 cases had a GSK-3β scoring intensity of (1) and 8 cases had a GSK-3β scoring intensity of (2). Using the Fisher's exact statistical test for comparison of grade 1/2 and grade 3 tumors, a P-value of 0,148 was calculated meaning that no statistically significant difference was achieved for the GSK-3β scoring intensity between grade 1/2 and grade 3 tumors. If the (0) and (1) scores were regrouped together, a P-value of 0,067 was reached between grade 1/2 and grade 3 tumors. Under the latter conditions, the difference of GSK-3β staining intensity between grade 1/2 and grade 3 tumors was still not statistically significant but a trend towards a higher GSK-3β staining intensity in grade 3 tumors could be observed. For the samples from the Chinese patients, the P-values obtained were too high to draw any conclusions. No obvious tendency for the GSK-3β scores was observed between the grade 1/2 tumors and the grade 3 tumors. Overall, the number of cases studied was too low to obtain any statistically significant differences in staining intensities according to the tumor

grade. However, analysis of the tumor samples from the German patients suggests that GSK-3β protein expression may increase with the tumor grade.

| German Patients | | | | |
|---|---|---|---|---|
| Tumour Grade | GSK-3β scoring intensity | | | Total |
| | 0 | 1 | 2 | |
| G1/G2 | 4 | 8 | 4 | 16 |
| G3 | 2 | 3 | 8 | 13 |
| Total | 6 | 11 | 12 | 29 |

| Chinese Patients | | | | |
|---|---|---|---|---|
| Tumour Grade | GSK-3β scoring intensity | | | Total |
| | 0 | 1 | 2 | |
| G1/G2 | 5 | 15 | 3 | 23 |
| G3 | 8 | 10 | 2 | 20 |
| Total | 13 | 25 | 5 | 43 |

**Figure IV - 26: Evaluation of immunohistochemical staining for GSK-3β.** Tumor samples from two ethnic populations were studied (29 German and 43 Chinese patients). For the German patients, 16 cases were grade 1/2 and 13 cases were grade 3. For the Chinese patients, 23 cases were grade 1/2 and 20 cases were grade 3. No statistically significant difference in GSK-3β staining intensity between the tumor grades was obtained using the Fisher exact test within both populations. However, a tendency toward an increase of GSK-3β staining intensity from the low grade to the high grade tumors was observed within the German patients.

# V - Discussion

We previously established functional links between GSK-3β activity and E2-dependent ERα activation and provided evidence that GSK-3β modulates ERα function by phosphorylation of relevant serine residues (Medunjanin *et al*., 2005). In the present work, the aim was to unravel the specific role of GSK-3 in ligand-dependent activation of ERα in human ER+ breast cancer cell lines. More precisely, we showed that GSK-3 not only plays a role in ERα activation by phosphorylating Ser-118, but also contributes significantly to the stability of ERα by complex formation in unstimulated cells. By this dual regulation of ERα, GSK-3 may link the nongenomic (cytoplasmic) and genomic (nuclear) effects of E2. Indeed, cytoplasmic GSK-3 is phosphorylated and inactivated upon E2 stimulation of cells, hypothetically, by nongenomic or rapid effects of E2. This releases ERα from its complex with GSK-3, allowing translocation of ERα into the nucleus. In this cellular compartment, E2-induced phosphorylation of the receptor at Ser-118 by a nuclear pool of GSK-3 will activate genomic action of ERα and stimulate transcription of ERα target genes.

This newly depicted signalling pathway, describing the regulation of ERα function and activity by GSK-3, may have some impact regarding breast cancer progression. Therefore, our actual aim is to study the relevance of GSK-3/ERα interaction according to the tumor grade. Preliminary data were obtained using GSK-3 immunostaining of formalin-fixed human tissue section.

## V - 1) Rapid effects of E2 treatment on the intracellular localisation and the phosphorylation status of ERα and GSK-3

After treatment of MCF-7 cells with E2, the phosphorylation status of GSK-3 and ERα and the intracellular localisation of ERα were rapidly altered, maximum effects being observed within less than 20 min after E2 stimulation. The genomic activity of E2-bound ERα can not explain these quick cellular responses and nongenomic effects of E2 were suggested to be responsible for these alterations. After E2 treatment, rapid translocation of ERα from the cytoplasm to the nucleus and nuclear phosphorylation of the receptor at Ser-118 were observed. Moreover, E2 treatment caused a rapid cytoplasmic GSK-3 phosphorylation leading to inactivation of the cytoplasmic fraction of the enzyme, while the

nuclear pool of GSK-3 was not affected. This allows nuclear GSK-3 to be a potential kinase phosphorylating nuclear ERα at Ser-118.

### V - 1.1) E2-induced translocation of ERα into the nucleus

In agreement with previous work from our group (Medunjanin *et al*., 2005; De Servi *et al*., 2005) and work from others (Horwitz and McGuire, 1978), ERα was detected in both the cytoplasm and the nucleus in serum-starved MCF-7 cells. After E2 treatment, translocation of the receptor from the cytoplasm to the nucleus of MCF-7 cells was observed. In resting MCF-7 cells, a smaller portion of this steroid receptor was reported to be localized in the cytoplasm (about 35 %) and the greater proportion of ERα was located in the nucleus; after E2 treatment, ERα was almost exclusively localized in the nucleus of these cells (Horwitz and McGuire, 1978). Moreover, E2-induced nuclear translocation of ERα is a prerequisite for the activation and function of ERα regarding genomic effects of the receptor, which are restricted to the nucleus of the cells.

### V - 1.2) E2-induced nuclear phosphorylation of ERα at Ser-118

E2-induced phosphorylation of Ser residues in the AF-1 domain of ERα is described as one of the most important features in signal transduction cascade leading to activation of the steroid receptor. In response to estradiol, ERα becomes predominantly phosphorylated in the AF-1 domain at Ser-118 and to a lesser extent at Ser-104 and Ser-106 (Lannigan, 2003). In agreement with these observations, we recently described that ERα was mainly phosphorylated at Ser-118 residue upon E2 treatment of MCF-7 breast cancer cells (Medunjanin *et al*., 2005). In the present work, we confirmed the strong E2-induced increase of ERα phosphorylation at Ser-118. After cellular fractionation, the data obtained suggest that this phosphorylation mainly occurs in the nucleus of MCF-7 cells. More precisely, a speckled nuclear accumulation of pSer118 ERα was first observed after E2 treatment. Then, pSer-118 ERα signal was detected in the whole nucleus. This suggests a time-scheduled organization of the molecular events in response to external stimuli and this is in agreement with the intranuclear mobility of ERα in association with nuclear matrix dynamics described by Matsuda *et al*. (2007).

A recent report (Valley *et al*., 2005), in agreement with the fact that Ser-118 phosphorylation was required for full ERα transcriptional activity (Lannigan, 2003), demonstrated that Ser-118 phosphorylation regulates transcriptional efficiency through the

differential recruitment of coactivators and transcriptional machinery to estrogen-responsive promoters. Work from our group, in agreement with these publications, also showed by co-transfection of constructs carrying ERα mutants and ERE-luciferase reporter plasmid in MDA-MB-231 ERα-negative cells that the S118A mutant construct activated ERE-dependent luciferase activity significantly less (about 3-fold) than the WT ERα construct (Medunjanin *et al*., 2005). Overall, phosphorylation of ERα at Ser-118 seems to be crucial for E2-induced ERα transcriptional activity. However, the protein kinases responsible for this phosphorylation are still under investigations. Work from our group (Medunjanin *et al*., 2005) suggested that ERα is a substrate for GSK-3, being phosphorylated at Ser-118. Therefore, GSK-3 may be one of the kinases involved in E2-induced ERα activation. This issue will be discussed in the part V - 2.2.3

### V - 1.3) Nongenomic effects of E2 regarding cytoplasmic GSK-3β activity

Since we suggested that GSK-3 plays a role in ERα activation, our first aim was to determine the effects of E2 on GSK-3 function. After E2 treatment, GSK-3α/β phosphorylation at Ser-21/9 was increased mainly in the cytoplasm, especially at the peri-nuclear site of MCF-7 cells. The kinetics of this E2-induced phosphorylation and consequent inactivation of GSK-3 kinase coincides with ERα translocation, which suggests a role for this kinase in the regulation of ERα translocation and implies that GSK-3 might be a gatekeeper to ERα nuclear entry. Nongenomic (cytoplasmic) effects of E2 may be responsible for this rapid phosphorylation. Unpublished work from our group has shown that PKB/Akt is rapidly activated by phosphorylation upon E2 treatment in MCF-7 cells. Interestingly, Greger *et al*. (2007) identified a so-called modulator of nongenomic action of estrogen receptor (MNAR) in MCF-7 cells and they suggested that phosphorylation of this novel scaffold protein promotes phosphorylation and activation of PI3K upon E2 stimulation, leading to increased phosphorylation of PKB/Akt. This mechanism could be responsible for the phosphorylation of the PKB/Akt-substrate GSK-3 in E2 treated cells.

Moreover, we observed an accumulation of ERα at the plasma membrane after 20 min E2 treatment. This event was transient and only minute amounts of ERα seemed to be involved. However, this accumulation may have relevant impacts on the E2 signalling pathway. Indeed, the aim of all signal transduction pathways is to amplify a signal to adapt the cells to the change of their environment. Only recently, a number of papers have been published on this topic to explain the modality of ERα membrane association and the rapid

effects (milliseconds to minutes) of this plasma membrane associated ERα in the non-genomic actions of estrogens (Vasudevan and Pfaff, 2007).

Taken together, these data suggest that E2 may bind to the plasma membrane-associated ERα and then activate the PI3K/Akt signalling pathway leading to rapid phosphorylation and inactivation of cytoplasmic GSK-3. Moreover, this does not exclude a role for putative nuclear GSK-3 regarding E2-induced ERα activation.

## V - 2) Sub-cellular roles of GSK-3β regarding E2 signalling pathway

As GSK-3 is involved in numerous signalling pathways, formation of intracellular GSK-3 pools was suggested. Cellular distribution of GSK-3 to the cytosol, mitochondria and nuclei enables local separation of GSK-3 functions (Ali *et al*., 2001; Jope and Johnson, 2004). In agreement with the assumption that a pool of GSK-3 related to a specific signalling pathway may be activated or inactivated upon a specific stimulus, we suggested that the regulation of ERα-mediated estrogen signalling was controlled by a dual action of GSK-3. Cytoplasmic GSK-3 may interact with and stabilize ERα under resting conditions and nuclear GSK-3 may be involved in ligand-dependent activation of the receptor (Medunjanin *et al*., 2005). Thus, GSK-3 may be the missing link and mediator between nongenomic and genomic effects of E2. Indeed, our results showed, in agreement with previous work form our group (De Servi et al., 2005; Medunjanin et al., 2005), that GSK-3 is present both in the cytoplasm and nucleus of the cells. Originally, GSK-3 was described mostly sequestered in cytosolic protein complexes. Recently, Meares and Jope (2007) identified a bipartite nuclear localization sequence (NLS) which is necessary for the nuclear accumulation of GSK-3β; and they discussed potential sub-cellular actions of the cytosolic and nuclear GSK-3. Additionally, E2 treatment did not seem to alter GSK-3 cellular localization suggesting that cytoplasmic and nuclear GSK-3 pools are distinct and work independently from each other in E2 treated cells.

To unravel the specific role of cytoplasmic and nuclear GSK-3 in ligand-dependent activation of ERα, several methodological approaches were considered. In the following, the results obtained will be discussed and their respective contributions to the model summarizing our findings and presented at the end of this part will be described.

### V - 2.1) Discrepancies of GSK-3β inhibitors regarding E2 signalling pathway

To study the role of GSK-3 regarding ERα signalling pathway, MCF-7 cells were treated with different GSK-3 inhibitors and their effects on E2-induced ERα translocation and nuclear phosphorylation at Ser-118 were monitored.

Jope (2003) showed that lithium ions reduce GSK-3 activity in two ways, both directly, as a competitive inhibitor of $Mg^{2+}$, and indirectly, by increasing phosphorylation of GSK-3. In agreement with this work, increased phosphorylation of GSK-3β at Ser-9 was observed in our experiments when cells were treated with LiCl. However, these data are at variance with another study. Indeed, Stambolic *et al*. (1996) described that LiCl did not inhibit GSK-3 by Ser-9 phosphorylation. They also suggest that LiCl inhibits GSK-3 kinase by mimicking the Wnt signalling pathway, inducing the accumulation of cytoplasmic β-catenin. Even if the molecular mechanisms leading to inhibition of GSK-3 by LiCl are still under discussion, lithium was unanimously shown to be a potent inhibitor of GSK-3. Regarding the ERα signalling pathway, LiCl did not influence the receptor protein level and did not alter the E2-induced ERα translocation. However, E2-induced Ser-118 phosphorylation of nuclear ERα was dramatically reduced. This suggests that GSK-3 phosphorylates ERα at Ser-118 upon E2 stimulation. However, the specificity of LiCl has been questioned. According to Coghlan *et al*. (2000), LiCl cause an acute inhibition of GSK-3 activity in cells, but LiCl also showed inhibitory effects on other protein kinases (as a low mM inhibitor of casein kinase-2 and p38 regulated/activated kinase). The LiCl-related decrease of ERα phosphorylation at Ser-118 after E2 treatment may therefore not exclusively be due to an inhibition of GSK-3.

To confirm the result obtained with LiCl, the recently described maleimide inhibitors $SB_4$ and $SB_2$ were used. These small-molecules are potent and selective inhibitors of GSK-3 (Cross *et al*., 2001). These cell permeable inhibitors act in an ATP competitive manner and 25 different serine/threonine and tyrosine protein kinases were tested to prove their high specificity regarding inhibition of GSK-3β kinase activity (Coghlan *et al*., 2000). However, while $SB_2$ inhibitor did not alter E2-induced ERα translocation and phosphorylation at Ser-118, treatment of MCF-7 cells with $SB_4$ inhibitor alone induced ERα translocation and increased Ser-118 phosphorylation of nuclear ERα. This unexpected observation implies unclarified mechanisms and unravels potential estrogenic properties of $SB_4$.

LiCl and maleimide inhibitors have all been shown to elevate the level of β-catenin in transformed cell lines and therefore might be expected to mimic the Wnt signalling pathway

and be potentially oncogenic (Cohen and Goedert, 2004). In our experiments, the oncogenic properties of these inhibitors were not observed. Indeed, LiCl and maleimides inhibited basal and E2-induced proliferation of MCF-7 cells. In breast cancer cells, these inhibitors may inhibit mammary tumor proliferation but the mechanisms are so far not understood and must be further investigated.

### V - 2.2) Effects of GSK-3 silencing on estrogen signalling pathway

To more specifically inhibit GSK-3, RNAi was used to silence both GSK-3 isoforms simultaneously or separately. The aim was to specifically target and therefore decrease the protein level of GSK-3 and to investigate if GSK-3 was involved in ligand-dependent activation of ERα.

#### V - 2.2.1) GSK-3 silencing disrupts ERα turnover

In absence of hormonal stimulation, a tightly regulated protein turnover allows the cells to maintain a balance between ERα protein synthesis and degradation leading to constant cellular level of the receptor (Reid *et al*., 2003). Our data clearly show that efficient silencing of GSK-3 in MCF-7 cells provokes a decrease of ERα protein level. First, we hypothesized that GSK-3 silencing-related decrease of ERα protein level was a consequence of a decrease in ERα transcription. In order to address this hypothesis, ERα mRNA was quantitated in cells transfected with GSK-3 siRNA. According to our results, an early transient decrease of basal ERα mRNA expression was observed (**Fig. IV - 10**). Adaptation of the cells to GSK-3 silencing on the one hand and to changes of the cell culture medium due to the experimental protocol on the other hand seemed to compensate this transient ERα mRNA decrease while the ERα protein level remained reduced in these cells. Furthermore, a downregulation of ERα protein level was observed upon E2 treatment and this downregulation was reinforced in cells transfected with GSK-3 siRNA. Once again, a reduction of ERα transcription was assumed regarding both E2 treatment and GSK-3 silencing. Interestingly, our data showed an E2-related decrease of ERα mRNA in control siRNA transfected cells, which can explain the decrease of the ERα protein level observed after E2 treatment. Indeed, an autologous downregulation pathway of ERα, which involves binding of liganded ERα to the promoter of its own gene, has been described (Saceda *et al*., 1988; Kaneko *et al*., 1993; Davis *et al*., 1999). By this mechanism, estrogens induce a decline in ERα mRNA and protein (Saceda *et al*., 1988). Surprisingly, GSK-3 silencing did not

further affect the autologous downregulation of ERα mRNA, although a tendency to a more pronounced decrease of ERα mRNA expression, which we cannot explain, was observed in cells treated with E2 for 48 h Overall, our data indicated that GSK-3 silencing did not significantly or only transiently alter basal and E2-related decrease of ERα mRNA levels at the time points investigated. These results suggest that the GSK-3 silencing-related ERα protein decrease might not be due to regulation of ERα transcription.

Then, inhibition of protein biosynthesis was considered as a putative mechanism leading to GSK-3 silencing-related decrease of ERα protein level. The translation inhibitor CHX was used to address this alternative. However, the use of this inhibitor did not support the hypothesis that GSK-3 silencing caused a decrease of ERα translation.

Our last hypothesis was that GSK-3 silencing resulted in increased ERα proteasomal degradation . The use of the proteasome inhibitor MG132 showed a significant rescue of ERα protein upon GSK-3 silencing both in unstimulated and E2-stimulated cells demonstrating a direct impact of GSK-3 on ERα proteasomal degradation. While these results do not exclude any modulation of ERα translation by GSK-3, GSK-3 is suggested to have an effect on ERα proteasomal degradation rather than on ERα biosynthesis. To confirm the GSK-3 silencing-related increase of ERα proteasomal degradation, immunoprecipitation of ERα was performed in presence of proteasome inhibitor MG132. Immunoblotting using anti-ubiquitin and ERα antibodies clearly showed an accumulation of ubiquitinated ERα and a rescue of ERα protein levels, respectively. These data showed that ERα degradation observed after GSK-3 silencing was due to proteasomal proteolysis. By this mechanism, GSK-3 silencing deregulates ERα protein turnover and decreases ERα protein level.

### V - 2.2.2) The docking properties of GSK-3 stabilizes ERα

Previous findings on direct interaction of the docking kinase GSK-3 with ERα raised the hypothesis that formation of an ERα/GSK-3 complex stabilizes ERα in the cytoplasm (Medunjanin *et al*., 2005). This hypothesis is corroborated by the observation presented in this report. It is shown that GSK-3 silencing using specific siRNA results in reduction of cellular ERα protein levels. Taken together, these findings permit the conclusion that GSK-3 protects ERα from proteasomal degradation and that complex formation between GSK-3 and ERα plays a crucial role in ERα protein stabilization and turnover. Complex formation involving GSK-3 has been shown to be important for stabilization of other proteins as well. In the Wnt

signalling pathway, active GSK-3 appears in a multiprotein complex that includes the transcription factor β-catenin and the scaffold protein axin (Frame and Cohen, 2001). Axin binding to GSK-3 and subsequent phosphorylation leads to stabilization of this protein (Yamamoto *et al*., 1999). In analogy to axin, the interaction of ERα with GSK-3 appears to be required for stabilization of the receptor.

In a separate independent approach, we confirmed that ERα protein levels were decreased upon GSK-3 silencing using miR expression vectors targeting GSK-3β. Transfection of xenopus GSK-3β constructs, in order to rescue the silencing of endogenous GSK-3, also resulted in the rescue the GSK-3-silencing related decrease of ERα protein content. This result does not only confirm the data described above regarding the stabilization of ERα by GSK-3, it also demonstrates the similarity in activity of the xenopus and the human GSK-3 kinase regarding ERα substrate. Interestingly, ERα protein rescue was achieved with both WT and DN xenopus GSK-3β constructs suggesting that the kinase dead GSK-3 may also form a complex with ERα. Therefore, GSK-3 kinase activity may not be required to stabilize ERα and the docking properties of GSK-3 protein may be responsible for complex formation with this receptor. However, reinforcement of ERα stabilisation by phosphorylation can not be excluded. Our first published model (Medunjanin *et al*., 2005) suggested a cytoplasmic GSK-3β-dependent phosphorylation of the Ser-102, 104, 106 motif of ligand-free ERα in resting cells. This phosphorylation may increase the stability of the complex formation between ERα and GSK-3β.

### V - 2.2.3) GSK-3 phosphorylates ERα at Ser-118 and is required for full E2-induced ERE-dependent ERα transcriptional activity

A second important role of GSK-3 regarding ERα function is phosphorylation of the receptor. *In vitro* kinase assay showed that ERα is a GSK-3 substrate being phosphorylated at Ser-118. This result is in agreement with *in vitro* kinase assays performed by our group using wild type and mutant (Ser- to Ala-) GST-ERα fusion proteins to identify the ERα serine residue(s) phosphorylated by GSK-3β (Medunjanin *et al*., 2005).

Since silencing of GSK-3 occurred in both the cytoplasm and the nucleus of the cells, and resulted in decrease of E2-induced ERα nuclear phosphorylation at Ser-118, we conclude that GSK-3 may phosphorylate this serine residue in the nuclei of MCF-7 cells. This is in agreement with data discussed in the part V - 1.2 that show a nuclear localization of E2-

induced Ser-118 phosphorylation of ERα. Another important aspect is that the ratio of pS118/ERα was reduced in GSK-3 silenced cells treated with E2. This clearly indicates that the reduction of Ser-118 phosphorylation observed after GSK-3 silencing was not the consequence of downregulation of ERα protein but particularly due to lack of GSK-3 kinase activity. However, residual pSer-118 signal was observed after GSK-3 silencing (**Figs. IV - 13, 14B and 17A**). While this was probably due to GSK-3 protein remaining after siRNA silencing, activity of another protein kinase cannot be excluded.

Moreover, data presented in this study showed that GSK-3 silencing resulted in a decrease of E2-induced ERE-dependent luciferase activity in MELN cells suggesting that GSK-3 silencing altered ERE-dependent transcriptional activity of ERα. After inhibition of the proteasome with MG132, the decrease of E2-induced luciferase expression was not restored whereas ERα protein level was rescued. It is possible that the ubiquitinated ERα forms may not have any transcriptional activity. Importantly, MG132 treatment alone did not significantly alter E2-induced luciferase activity in cells transfected with either control siRNA or GSK-3 siRNA. These results are at variance with a study published previously (Reid *et al*., 2005) which reported a reduction of E2-induced ERE-dependent luciferase expression by MG132 treatment. The different findings can be explained by the different experimental settings. To minimize the side effects of MG132 in our study, we used a low dose of proteasome inhibitor for a short treatment period when measuring E2-induced ERE-luciferase activity in MELN cells.

The results obtained from MELN cells transfected with GSK-3β constructs showed that overexpression of GSK-3β increased E2-induced ERα transcriptional activity. Similar results were obtained for the GSK-3β WT and the GSK-3β constructs inactive regarding GSK-3 primed substrates (i.e. R96A and R96K GSK-3β). Considering that Ser-118 phosphorylation is a crucial step for E2-induced ERα transcriptional activity (see part V - 1.2), these results suggested that ERα may be phosphorylated at Ser-118 by GSK-3 in a non-primed manner. This observation is in agreement with the comparative alignment between sequences of ERα motif at Ser-118 and sequences of motifs of non-primed substrates such as Tau and cyclin D1 (Medunjanin *et al*., 2005).

It is concluded that ERα is a non-primed substrate of GSK-3, being activated by phosphorylation at Ser-118 upon E2 stimulation. Moreover, the data demonstrate that GSK-3 is required for full E2-induced ERE-dependent ERα transcriptional activity.

### V - 2.2.4) GSK-3 is required for E2-induced expression of endogenous ERα target genes

GSK-3 silencing resulted in a decrease of E2-induced expression of pS2 and progesterone receptor suggesting that GSK-3 is required for transcriptional expression of estrogen responsive genes. Although the regulation of ERα target genes via the sole classical ERE binding is under debate, pS2 gene shows a consensus ERE palindromic sequence, while progesterone receptor gene seems to have an alternative consensus ERE sequence (Klinge, 2001). Therefore, GSK-3 may modulate a broad-range of estrogen responsive genes and not only ERα target genes with the sole consensus ERE sequence.

### V - 2.2.5) Model for the effects of GSK-3 silencing regarding ERα

In previous work, our group has proposed a model for the potential function of GSK-3 in ERα activation (Medunjanin *et al*., 2005). This model is now complemented by including the present findings (**Fig. V - 1A**). In unstimulated cells, a complex between active GSK-3 and ERα stabilizes the receptor in the cytoplasm. Upon E2 treatment, phosphorylation of GSK-3α/β at Ser-21/9 residues results in inhibition of the kinase. Consequently, ERα is released and translocates into the nucleus where it is phosphorylated by active nuclear GSK-3 at Ser-118 leading to full transcriptional activity. Eventually, E2 signal is switched off by ubiquitination and proteasomal degradation of ERα. The effects of GSK-3 silencing are described in **Fig. V - 1B**. The lack of GSK-3 results in reduction of ERα stabilization triggering its ubiquitination and degradation by the proteasome. As a consequence, ERα protein content is decreased. Moreover, E2-induced Ser-118 phosphorylation of the remaining ERα is reduced due to lack of GSK-3 kinase activity in the nucleus, which results in decrease of E2-induced ERα transcriptional activity.

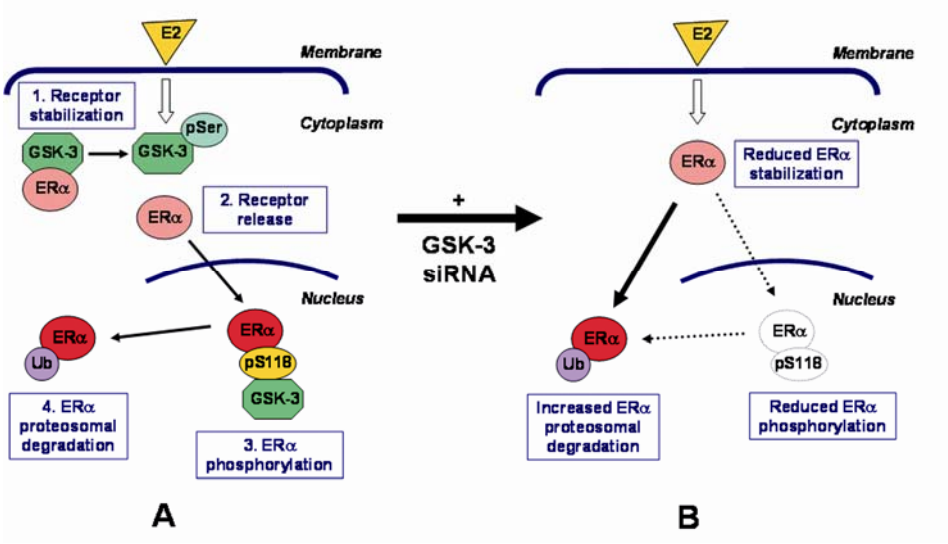


**Figure V - 1: Model for the effects of GSK-3 silencing regarding ERα.** ***Panel A.*** In unstimulated cells ERα is stabilized by interaction with active GSK-3 in the cytoplasm (*1*). Treatment of cells with E2 results in phosphorylation and inactivation of GSK-3, ERα release (*2*), translocation of ERα into the nucleus where it is phosphorylated by active nuclear GSK-3 at Ser-118 (*3*). Eventually, E2 signal is switched off by ubiquitination and proteasomal degradation of ERα (*4*). ***Panel B.*** Lack of GSK-3 results in reduction of ERα stabilization triggering its ubiquitination and proteasomal degradation and in inhibition of E2-induced ERα phosphorylation at Ser-118 (Grisouard *et al*., 2007).

## V - 3) GSK-3 expression in breast cancer of different grades

Data discussed so far have been obtained from cell culture models. The physiological relevance of the results remains to be clarified. It is of utmost interest whether ERα/GSK-3 interaction occurs *in vivo* and triggers similar signals as observed in cell culture. If this proofs true ERα/GSK-3 could contribute to progression of estrogen-dependent cancers. Preliminary data are available from GSK-3 immunostaining experiments of formalin-fixed breast tissue sections taken from two ethnically different groups, namely German and Chinese patients. The results suggest a tendency toward increased GSK-3 expression in grade 3 tumors in comparison with the grade 1/2 tumors within the German group of patients. Since grade 3 tumors show a more aggressive phenotype and spread more easily, these first results suggest a role for GSK-3 in breast cancer progression and in increase of breast tumor malignancy. However, the statistical evaluation of the data using the Fisher's exact test did not prove this tendency. The number of cases investigated must be increased to draw statistical conclusions regarding GSK-3 expression pattern in breast tumorigenesis. Furthermore, a correlation with ERα expression must be provided. Moreover, the tendency observed for the German patients was not confirmed for the Chinese patients. While differences between the two ethnic

populations cannot be excluded, variations in the procedures used to supply the tissue specimens may explain possible discrepancies in the GSK-3β immunostaining of the tissue sections between the two groups. More precisely, the fixation step before embedding of the tissue, which is crucial for antigen recognition by the GSK-3β antibody, may have differed.

In conclusion, GSK-3 was described as a key regulator of ligand-dependent activation of ERα and a role for GSK-3 in breast tumor progression and malignancy was suggested. These data may consider GSK-3 or better GSK-3/ERα interaction as a pharmaceutical target to inhibit estrogen-dependent growth of breast cancer cells and to prevent breast tumor progression. GSK-3 inhibitors were first synthesized to treat diabetic patients and to normalize their blood glucose levels. These inhibitors are now under investigation for the treatment of neurological disorders such as Alzheimer's disease (Cohen and Goedert, 2004). Additionally, several recent publications suggested a potential new therapeutic application for GSK-3 inhibitors in prostate cancer (Mazor *et al*., 2004), colorectal cancer (Shakoori *et al.*, 2005; Shakoori *et al*., 2007), ovarian cancer (Cao *et al*., 2006) and pancreatic cancer (Ougolkov *et al*., 2006; Garcea *et al*., 2007), among others. Therefore, GSK-3 inhibitors or modulators should be considered to treat breast cancer. However, sustained GSK-3β inhibition was suggested as a hallmark of tumorigenesis. *In vivo*, transgenic mice overexpressing the DN-GSK-3β under the mouse mammary tumor virus (MMTV) develop mammary tumors with overexpression of β-catenin and cyclin D1 (Farago *et al*., 2005). These data suggest that the inhibition of GSK-3 activity mimics the Wnt pathway and could promote mammary tumors.

## V - 4) Outlook

Prior to consider GSK-3 inhibitors as a possible therapeutic approach for ER+ breast cancer, further experiments need to be performed in order to investigate the efficiency and the potential side effects of promising GSK-3 inhibitors in the treatment of breast cancer. GSK-3 is involved in various signalling pathways and GSK-3 regulates a wide range of cellular processes including metabolism, differentiation, growth, motility and apoptosis. New insights into the GSK-3 signalling pathways will especially help to select novel therapeutical approaches. New findings regarding GSK-3 interaction particularly with ERα and β-catenin

substrates and improvement in the understanding of the cross-talks between the related signalling pathways will notably permit to target the estrogenic pathway without side effects on the Wnt pathway, thus avoiding harmful side effects of possible GSK-3 treatment.

The perspective of GSK-3β mutants selectively affecting ERα and putatively disrupting GSK-3/ERα interaction will be preferentially studied. In this work, we described a role for GSK-3 in ligand-dependent activation of ERα. We showed that GSK-3 protects ERα from proteasomal degradation and is involved in full transcriptional activation of the receptor. However, the GSK-3 domains or amino acids specifically involved in the interaction with ERα remain to be identified to determine their specific role either in ERα stabilization or in ERα phosphorylation and transcriptional activation, or in both of these newly depicted GSK-3 functions. Therefore, it will be of interest to investigate the effects of the overexpression of various functional mutants of xenopus GSK-3β in cells transfected with miR targeting endogenous human GSK-3β. While we showed that the docking properties of GSK-3 were important for the stabilization of ERα, more work using the WT and DN xenopus GSK-3β constructs will be performed to further analyse the involvement of GSK-3 in E2-induced ERα phosphorylation and transcriptional activity. Additionally, other GSK-3β constructs will be tested. They include the S9A or CA GSK-3β construct, the R96A and R96K GSK-3β constructs, which are inactive regarding phosphorylation of primed-phosphorylated substrates by GSK-3, the GSK-3β dN (delta N) showing a N-terminal deletion of 63 amino acids, as well as the GSK-3β GR (V267G/E268R) construct which selectively disrupts axin-GSK-3 interaction (Fraser *et al*., 2002).

The next step will be to confirm *in vivo* the findings obtained in cancer cell lines. The effect of GSK-3 inhibitors on the progression of ERα-dependent mammary tumors will be investigated after orthotopical injection of ER+ breast cancer cells in nude mice. Similarly, the effects of GSK-3 inhibitors on transgenic mice expressing genes leading to the development of mammary gland adenocarcinomas, such as MMTV-Wnt1 transgenic mice (Tsukamoto *et al*., 1998) may be studied and especially the ERα-dependent progression of these tumors should be investigated. Other transgenic mouse models such as mice with constitutive knock-in of homologous GSK-3α S21A/GSK-3β S9A (MacManus *et al*., 2005) and mice overexpressing the DN-GSK-3β in the mammary gland (Farago *et al*., 2005) could be used to study GSK-3/ERα interaction *in vivo*.

Finally, the impact on human breast tumorigenesis of the GSK-3/ERα signalling pathway depicted in cancer cell lines needs further clarification. Preliminary immunohistochemistry data of tumor tissues from surgically-operated patients suggested a role for GSK-3 in breast tumor progression and malignancy. We will extend this study to a larger cohort of patients. . The increase of cases studied will also permit a statistical analysis of GSK-3 expression according to ERα expression within a group of specific tumor grade. Furthermore, the protein levels of GSK-3 and ERα do not indicate their functional status. Therefore, the phosphorylation status or inactivation level of GSK-3 and the phosphorylation status or activation level of ERα will be investigated in the collected cases. The data obtained from immunohistochemistry will be correlated with the clinical outcome of the patients.

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# Publications related to my PhD thesis

**Grisouard J**., S. Medunjanin, A. Hermani, A. Shukla and D. Mayer. (2007). Glycogen synthase kinase 3 protects estrogen receptor alpha from proteasomal degradation and is required for full transcriptional activity of the receptor. *Mol. Endocrinol.,* in press (**Epub ahead of print July 3, 2007 as doi: 10.1210/me.2007-0129**).

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# Abbreviations

- AC — Adenylyl Cyclase
- ADP — Adenosine Diphosphate
- AF-1 — Transcriptional Activation Function-1
- AF-2 — Transcriptional Activation Function-2
- AGC — Group of protein kinases A, G and C
- Ala (A) — Alanine, amino acid
- AMP — Adenosine Monophosphate
- AP-1 — Activator Protein-1
- APC — Adenomatous Polyposis Coli
- APP — Amyloid Precursor Protein
- Arg (R) — Arginine amino acid
- ATP — Adenosine Triphosphate
- bp — base pair
- BSA — Bovine Serum Albumine
- BT474 — human breast carcinoma cell line, ER+
- CA — Constitutively Active
- $Ca^{2+}$ — Calcium
- CaMK — Group of protein kinases regulated by CaM
- CaM — Calcium/calmodulin
- cAMP — cyclic AMP
- CAT scans — Computerized Axial Tomography
- Cdk — Cyclin-dependent kinase
- Cdk2 — Cyclin dependent kinase 2
- cDNA — complementary or copy DNA
- C/EBPα — CCAAT/Enhancer Binding Protein α
- cGMP — cyclic GMP
- CHX — Cycloheximide
- CK2 — Casein Kinase 2
- CNS — Central Nervous System
- $CO_2$ — Carbon Dioxide
- COX-2 — Cyclooxygenase-2
- cpm — counts per minute
- CREB — cAMP Response Element Binding protein
- CTD kinases — Carboxy-Terminal Domain kinases
- c-Src — Cellular homologue of v-Src
- DAB — 3,3'-Diaminobenzidine
- DBD — DNA-Binding Domain
- DCC — Dextran-Coated Charcoal
- DCIS — Ductal Carcinoma In Situ
- DHEA — Dehydroepiandrosterone
- DMEM — Dulbecco's Modified Eagle's Medium
- DMSO — Dimethyl Sulphoxide
- DN — Dominant Negative
- DNA — Deoxyribonucleic Acid
- dsDNA — double-stranded DNA
- dNTPs — deoxynucleotides Triphosphate
- DTT — Dithiothreitol
- DVL — Dishevelled
- DYRK — Dual-specificity Tyrosine-phosphorylated and Regulated Kinase
- ECL — Enhanced Chemiluminescence
- EDTA — Ethylen diaminetetraacetic Acid
- EGF — Epidermal Growth Factor
- EGFR — Epidermal Growth Factor Receptor
- eIF2B — eukaryotic Initiation Factor 2B
- ER — Estrogen Receptor
- ERα — Estrogen Receptor alpha

- ERβ Estrogen Receptor beta
- ErbB EGFR family
- ERE Estrogen Response Element
- ERK Extracellular signal-Related Kinase
- ER- ERα negative
- ER+ ERα positive
- E2 17β-Estradiol
- FCS Foetal Calf Serum
- FGFR Fibroblast Growth Factor Receptor
- Fos nuclear DNA binding protein (product of the c-Fos proto-oncogene)
- FRAT Frequently Rearranged in Advanced T-cell lymphomas
- FSH Follicle Stimulating Hormone
- Fwd primer Forward primer
- Fyn member of the Src tyrosine kinase family
- GAPDH Glyceraldehyde-3-Phosphate Dehydrogenase
- GF Growth Factor
- GFP Green Fluorescence Protein
- GMCC Protein kinases group including GSK, MAPK, Cdk and CTD kinases.
- GMP Guanosine Monophosphate
- GnRH Gonadotrophin Releasing Hormone
- GS Glycogen Synthase
- GSK Glycogen Synthase Kinase
- GSK-3 Glycogen Synthase Kinase-3
- GSK-3α Glycogen Synthase Kinase-3alpha
- GSK-3β Glycogen Synthase Kinase-3beta
- GST Glutathione-S-Transferase
- h hour
- H & E Hematoxylin and Eosin
- HCl Hydrochloric acid
- HEPES 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic acid
- HEPES/KOH HEPES/Potassium hydroxide
- HER2 Human epidermal growth factor receptor 2 or ErbB2
- HRP Horseradish Peroxidase
- HRT Hormone Replacement Therapy
- HSF-1 Heat Shock Factor-1
- IDC Invasive Ductal Carcinoma
- IGF-1 Insulin-Like Growth Factor-1
- IGFR Insulin Growth Factor Receptor
- IgG Immunoglobulin G
- IHC Immunohistochemistry
- ILC Invasive Lobular Carcinoma
- IL-6 Interleukin-6
- IRS-1 Insulin Receptor Substrate-1
- JNK c-Jun N-terminal kinase
- Jun nuclear DNA binding protein (product of the c-Jun proto-oncogene)
- KCl Potassium Chloride
- kDa kilo Dalton
- l litre
- LacZ bacterial gene encoding the β-galactosidase
- LAR Luciferase Assay Reagent
- LB Luria Broth Bertani (culture medium for bacteria)
- LBD Ligand Binding Domain
- LCIS Lobular Carcinoma In Situ
- LEF Lymphoid-Enhancer Factor
- LH Luteinising Hormone
- LHRH Luteinising Hormone-Releasing Hormone
- LiCl Lithium Chloride
- Luc Luciferase
- Lys (K) Lysine, amino acid
- m milli

- M Molar
- MAP Microtubule Associated Protein
- MAPKAP-K1 MAPK-Activated Protein Kinase 1 or p90Rsk
- MAPK Mitogen-Activated Protein Kinase
- MCF-7 human breast carcinoma cell line, ER+
- MDA MB 231 human breast carcinoma cell line, ER-
- MELN MCF-7 cells stably transfected with ERE-luciferase reporter plasmid
- MEK MAPK/ERK kinase
- $Mg^{2+}$ Magnesium
- $MgCl_2$ Magnesium Chloride
- MG132 Proteasome inhibitor
- $MgSO_4$ Magnesium sulfate
- µ micro
- min minutes
- miR micro RNA
- MMTV Mouse Mammary Tumor Virus
- $MnCl_2$ Manganese Chloride
- mRNA messenger RNA
- mTOR mammalian Target Of Rapamycin
- Myc protein inducing cell growth and encoded by the c-Myc proto-oncogene
- NaAc Sodium Acetate
- NaCl Sodium Chloride
- $Na_2CO_3$ Sodium Carbonate
- NaF Sodium Fluoride
- $Na_2HPO_4$ Di-sodium-hydrogen-phosphate
- $NaH_2PO_4$ Sodium dihydrogen phosphate
- $NaN_3$ Sodium azide
- NaOH Sodium hydroxide
- $Na_3VO_4$ Sodium orthovanadate
- NCAM Neural Cell-Adhesion Protein
- NCI National Cancer Institute (USA)
- NFAT Nuclear Factor of Activating T cells
- NFκB Nuclear Factor kappa B
- NGF Nerve Growth Factor
- NIH National Institutes of Health (USA)
- NLS Nuclear Localization Signal
- nm nanometres
- O.D. Optical Density
- ONPG 2-Nitrophenyl-β-D-Galactopyranoside
- OPK group of Other Protein Kinases
- OSM Oncostatin M
- O/N Overnight
- PAGE Polyacrylamide Gel Electrophoresis
- PBS Phosphate Buffered Saline
- PCR Polymerase Chain Reaction
- PDGFR Platelet-Derived Growth Factor Receptor
- PDK1 3-Phosphoinositide-Dependent Protein Kinase-1
- PFA Paraformaldehyde
- PGE2 Prostaglandin E2
- PKA Protein Kinase A
- PKB Protein Kinase B or Akt
- PKC Protein Kinase C
- PKCδ PKC delta
- PI3K Phosphatidylinositol 3-Kinase
- PIK3CA Gene encoding the PI3K Catalytic subunit (p110 Alpha)
- PMSF Phenylmethylsulphonyl Fluoride
- PR Progesterone Receptor
- pS2 estrogen-responsive gene (TIFF1 or Trefoil Factor 1)
- PTK Protein Tyrosine Kinase
- PVDF Polyvinylidenfluoride

- p38 MAPK Mitogen Activated Protein Kinase p38
- rcf Relative Centrifugal Force
- RDδ Regulatory Domain of PKCδ
- Rev primer Reverse Primer
- RNA Ribonucleic acid
- RNAi RNA interference
- RNase Ribonuclease
- RPA / p34 Replication Protein A or Human Single-Stranded DNA-Binding protein (HSSB)
- rpm revolutions per minute
- RPMI Roswell Park Memorial Institute cell culture medium
- Rsk Ribosomal S6 Kinase or MAPKAP-K1
- RT Room Temperature
- RTK Receptor Tyrosine Kinase
- RT-PCR Reverse Transcriptase- Polymerase Chain Reaction
- $SB_2$ GSK-3β inhibitor SB216763 (from Glaxo-Smith-Kline)
- $SB_4$ GSK-3β inhibitor SB415286 (from Glaxo-Smith-Kline)
- SBR Scarff-Bloom-Richardson (tumor grading)
- SDS Sodium Dodecyl Sulfate
- sec seconds
- SEM Standard Error of the Mean
- Ser (S) Serine, amino acid
- SERD Selective Estrogen Receptor Destabilisator
- SERM Selective Estrogen Receptor Modulator
- shRNA short hairpin RNA
- Shc Src-homology adaptor complex
- siRNA small interfering RNA
- SNPs Single-Nucleotide Polymorphisms
- SOC bacterial culture media
- Sp-1 Specificity Protein 1
- SYBR SYBR green (DNA intercalating dye)
- TAE Tris Acetate EDTA buffer
- Taq Thermus Aquaticus
- TBS Tris-buffered saline
- TBS/T TBS/Tween 20
- TCF T-cell factor family of transcription factors or LEF
- TE Tris-EDTA buffer
- TEMED N, N, N', N'-Tetramethyl ethylenediamine
- TFIIH associated kinase complex
- T47D human breast carcinoma cell line, ER+
- Thr (T) Threonine, amino acid
- Tm melting Temperature
- TNFα Tumor Necrosis Factor alpha
- TNM Tumor-Nodes-Metastasis (cancer staging)
- TPA 12-*O*-Tetradecanoylphorbol-13-Acetate
- Tris trishydroxymethylaminomethane
- Tyr (Y) Tyrosine, amino acid
- U Unit of the enzyme activity
- UV Ultraviolet
- VEGFR Vascular Epithelial Growth Factor Receptor
- v-Src Oncoprotein encoded by Rous sarcoma virus
- v/v volume per volume
- Wnt vertabrates secreted glycoprotein equivalent to the Wingless in drosophila
- WT Wild Type
- w/v weight per volume
- ZAK1 Dictyostelium protein kinase

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# Erklärung

Hiermit erkläre ich, Jean Grisouard, daß ich die vorliegende Dissertation selbst angefertigt habe und keine anderen als die aufgeführten Hilfsmittel und angegebenen Quellen benutzt habe. Ich habe die vorliegende Arbeit an keiner anderen in- oder ausländischen Fakultät eingereicht.

Heidelberg, the 23rd of August 2007,

Jean Grisouard.

# Curriculum Vitae

Name: **Jean Grisouard**
Date of Birth: 09.11.1981
Nationality: French
Tel: + 49 6221 63 56 51
Address: Rheinstrasse 35
69126 Heidelberg
Germany
E-mail: **jean.grisouard@laposte.net**

## EDUCATION

**2004-2007** **PhD - German Cancer Research Centre (DKFZ)**
Heidelberg, Germany

**2001-2004** **BSc Physiology and Pharmacology** (BSc Hons II.i)
The Nottingham Trent University (TNTU, Nottingham, England)

**Diploma in Professional Skills** (with distinction)
One-year placement at **Novartis** (Basel, Switzerland)

**1999-2001** **Diplome Universitaire de Technologie (D.U.T.) Applied Biology**, option Biological and Biochemical laboratory analysis
I.U.T. (Dijon, France)

**1996-1999** **Baccalaureat**, scientific option ("A levels", Dijon, France)

## WORK EXPERIENCE

**Sept/2004 - Present** **PhD - Hormones and Signal Transduction Group (DKFZ)**

**Title:** Interaction between glycogen synthase kinase-3 and estrogen receptor alpha in ligand-dependent activation of the receptor

**Methodological Approach:** Molecular biology (plasmid cloning and mutagenesis, RNAi), Biochemistry (kinase assay, immunoprecipitation and western blot, luciferase and β-gal assays), Cell culture (transfection, lysate and fractionation), Immunofluorescence and Immunohistochemistry.

**Publications:**

**Grisouard, J.**, Medunjanin, S., Hermani, A., Shukla, A., and Mayer, D. (2007). Glycogen synthase kinase-3 protects estrogen receptor-α from proteasomal degradation and is required for full transcriptional activity of the receptor. *Molecular Endocrinology*, in press (July 3, **Epub ahead of print**).

**Grisouard J.**, Trauth, S., Hermani, A. and Mayer, D. (2006). Glycogen synthase kinase-3β (GSK-3β) as a key component of estradiol signalling pathway. *European Journal of Cancer, supplements* **4**, 42.

Medunjanin, S., Hermani, A., De Servi, B., **Grisouard, J.**, Rincke, G., and Mayer, D. (2005). Glycogen synthase kinase-3 interacts with and phosphorylates estrogen receptor-α and is involved in the regulation of receptor activity. *Journal of Biological Chemistry* **280**, 33006-33014.

**Congresses/Conferences:**

- **Poster Presentations:** *14th International AEK Cancer Congress* (Frankfurt, Germany, February 2007); *Symposium 'Targeting the Kinome'* (Basel, Switzerland, December 2006); *31st Symposium on Hormones and Regulation: Cancer Cell Signalling* (Mont Sainte-Odile, France, September 2006)

- **Poster Award:** *2nd International Conference 'Molecular Staging of Cancer'* (Heidelberg, Germany, June 2006)

- **Oral Presentation** (May 2005) and **Poster Presentation** (May 2006) at the *9th and 10th Graduate Seminars for DKFZ International PhD Student Program* (Weil der Stadt, Germany)

**Jan/2004 - May/2004** **Research Project** (Biomedical Research Centre, TNTU).
Title: UDP-Glucose receptor expression and function in lymphocytes

Methodological Approach: Primary T and B lymphocyte cells isolation, cell culture proliferation and RNA analysis

**Aug/2002 - July/2003** **Placement** (Oncology-Pharmacology unit, Novartis)
Title: MTLn3 luc 9D rat breast cancer cell line characterisations

Methodological Approach:
*In vitro*: cell culture proliferation, lysate and fractionation; apoptosis DNA fragmentation and caspase activity; luciferase reporter assay
*In vivo*: animal housing, breast tumor cells inoculation, tumor volume monitoring, doxorubicin treatment and Xenogen IVIS imaging

Additional Information: Introductory course in laboratory animal science (17th/22nd March 2003, Zürich, Switzerland)

**July/2001 - Sept/2001** **Lab assistant** (Laboratoire Augey, Is sur Tille, France).
Human blood analysis from taking blood sample to analysing it.

**April/2001 - June/2001** **Training** (Medical Biochemistry Lab, INSERM, Dijon, France). Flow Cytofluorimetry Measurement of Cholesterol Oxides Accumulation in Rat Smooth Muscle Cells.

## OTHER QUALIFICATIONS

Languages: French (fluent), English (fluent) and German (Advanced level)
Computer software: Familiar with Microsoft Office and Internet

## INTERESTS

Sports: Mountain biking, cycling, kayaking, trekking and football (German League)
Travels: England, Switzerland, Germany for studies and professional trainings. Italy, Spain, Chile and Peru for cultural interests

## REFERENCES

Personal references available upon request.